A HIGH-FAT DIET ALTERS THE PHENOTYPE OF DIABETIC NEUROPATHY

BY

Brianne Lynn Guilford

Submitted to the graduate degree program in Anatomy and Cell Biology and the Graduate Faculty of the University of Kansas in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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Chairperson Douglas E. Wright, Ph.D.

_____

Julie A. Carlsten Christianson, Ph.D.

_____

Paige C. Geiger, Ph.D.

_____

Lisa A. Stehno-Bittel, P.T., Ph.D.

_____

Russell H. Swerdlow, M.D.

Date Defended: April 9th, 2013

The Dissertation Committee for Brianne Lynn Guilford certifies that this is the approved version of the following dissertation:

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Chairperson Douglas E. Wright, Ph.D.

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Abstract

Diabetic neuropathy is a principal chronic complication of both type 1 and type 2 diabetes and affects over half of diabetic patients. This debilitating disease presents with a dichotomous phenotype such that affected patients can experience both negative and positive sensory symptoms including chronic numbness, altered sensitivity to pain or touch, and impaired proprioception. In light of long withstanding evidence that hyperglycemia is the primary cause of diabetic neuropathy, evidence from several large clinical studies indicate metabolic defects such as a poor lipid profile are linked with neuropathy development and progression, independent of glycemic control. Consequently, dyslipidemia has recently been identified as an independent risk factor for diabetic neuropathy.

The purpose of this body of work was to test the effects of diet and dyslipidemia on the development and progression of diabetic neuropathy and identify potential mechanisms underlying the pathogenesis of high-fat diet induced neuropathy. Initial studies characterized the effects of a high-fat diet on neuropathy progression and phenotype in nondiabetic and streptozocin (STZ) induced (type 1 model) diabetic mice. STZ-induced diabetic C57Bl/6 mice fed a high-fat diet developed dyslipidemia and a painful neuropathy (mechanical hyperalgesia) instead of the insensate neuropathy (mechanical insensitivity) that normally develops in this mouse strain. Nondiabetic mice fed the high-fat diet also developed dyslipidemia and mechanical hyperalgesia. These findings are particularly important because it suggests that diet may modulate diabetic neuropathy phenotype.

Second, mitochondrial dysfunction, inflammation, and peripheral nervous system insulin resistance were investigated as potential mechanisms underlying the pathogenesis of high-fat diet induced painful neuropathy. Results from these studies suggest that none of these mechanisms

are driving the robust behavioral phenotype observed in high-fat-fed nondiabetic and streptozocin-induced diabetic mice.

Finally, exercise ameliorated the detrimental effects of a high-fat diet on body weight, circulating glucose and insulin levels, and skeletal muscle insulin resistance. These results reinforced the importance of exercise in preventing or reversing symptoms of diabetes. Together, these studies provide strong evidence for the influence of lifestyle factors, including diet and physical activity, on metabolic defects and neural complications associated with diabetes.

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CHAPTER 1

Introduction

Diabetes Mellitus

Diabetes mellitus is a chronic metabolic disorder characterized by insufficient production or utilization of insulin. Insulin deficiency, resulting in inadequate cellular glucose uptake and utilization, causes hyperglycemia, excess urine production, and increased fluid intake, the hallmark symptoms of this disease. In 2011, the American Diabetes Association reported that 25.8 million children and adults in the United States (8.3% of the population) have diabetes although one quarter of those affected are undiagnosed cases [1]. The prevalence of diabetes continues to increase with 1.9 million new cases of diabetes diagnosed in individuals over 20 years of age per year [1]. Type 1 diabetes affects only 5-10% of diabetic patients and is caused by deficient insulin production due to autoimmune destruction of pancreatic beta cells. Type 2 diabetes, accounting for 90-95% of diabetic cases, is caused by reduced insulin sensitivity in muscle and adipose resulting in impaired glucose uptake in these tissues [1]. Type 1 diabetes is typically associated with a younger age of onset while older age, obesity, and physical inactivity are associated with and considered risk factors for type 2 diabetes [1]. Both type 1 and type 2 diabetes are associated with serious complications including heart disease, retinopathy, nephropathy, and neuropathy, which can lead to stroke or heart attack, vision loss, kidney failure, and limb amputations, respectively. Diabetes and its associated complications have a serious economic impact accounting for $116 billion in medical costs and $58 billion in indirect costs (disability, work loss, premature mortality) each year [1]. Further illustrating the astronomical impact of this disease, diabetes was the 7th leading cause of mortality in the United States in 2007 [2].

Diabetic Neuropathy

Diabetic neuropathy is the most common and debilitating complication of both type 1 and type 2 diabetes and encompasses several neuropathic syndromes [3]. Diabetic neuropathies can be classified into two broad categories: diffuse and focal neuropathies [3, 4]. Diffuse neuropathies are more common and typically chronic and progressive in nature while focal neuropathies are less common, usually acute in onset, and often self limited [3]. The diffuse neuropathies include distal symmetric sensorimotor polyneuropathy and diabetic autonomic neuropathy [5]. Distal symmetric sensorimotor polyneuropathy, the most common and widely recognized form of diabetic neuropathy, is characterized by both sensory and motor nerve deficits; however sensory dysfunction is the predominant feature of this neuropathy [3]. In the literature, several names are used interchangeably with distal symmetric sensorimotor polyneuropathy, and diabetic peripheral neuropathy is one of the most common synonyms. Approximately 70% of diabetic patients will develop peripheral neuropathy [4, 6, 7]. Affected patients can experience a large spectrum of sensory symptoms including chronic numbness, altered sensitivity to pain or touch, and impaired proprioception [6, 8, 9]. There is no definitive cure for this debilitating disease and symptomatic treatments have shown limited success [10]. This body of work is focused on studying distal symmetric sensorimotor polyneuropathy, which will be referred to as diabetic neuropathy from this point forward.

Diabetic neuropathy often initially presents as an insensate phenotype that later progresses to a painful phenotype [11]. In more advanced stages of this disease, symptoms may progress to include motor weakness and impaired proprioception, which can lead to gait disturbances and increased risk for falls [4]. Loss of sensation in the extremities frequently leads to foot ulcers, thus making diabetic neuropathy the leading cause of amputations [12]. The type

of symptoms a patient experiences depends on the type of nerve fibers affected [3]. Large fiber disease is associated with impaired proprioception and response to light touch while small fiber disease impairs pain and temperature perception [3].

Pathogenesis

Regardless of the nerve fiber type affected or neuropathy subtype, a dying back-type distal axon degeneration is the common underlying feature associated with diabetic neuropathy [13]. In addition, axon loss may be accompanied by other axonal abnormalities including segmental demyelination and reduced nerve regeneration capacity [14-16]. It is thought that nerve dysfunction and degeneration leads to sensorimotor deficits, reduced nerve conduction velocities, and decreased epidermal innervation, all of which are characteristic signs of diabetic neuropathy in human patients and animal models [10, 17]. In the setting of diabetes, evidence points to hyperglycemia as the initiation factor that leads to a variety of metabolic insults that ultimately damage peripheral sensory neurons. Hyperglycemia plays a key role in the development and progression of diabetic neuropathy [3, 11, 17-19], and a combination of multiple etiologies, each stemming from the initial insult of hyperglycemia, are likely responsible for axonal degeneration leading to the various types of neuropathy in diabetic patients [3, 18, 19]. Hyperglycemia has been shown to lead to oxidative stress, mitochondrial dysfunction, increased glucose flux through the polyol pathway, advanced glycation end product (AGE) formation [3, 6, 9, 18], disrupted insulin signaling, inflammation, and diminished neurotrophic support [20-22]. Each of these mechanisms are currently being studied in rodent models of diabetic neuropathy. In addition, dyslipidemia has recently

been proposed as an important risk factor that may increase a diabetic patient's risk of developing neuropathy [5, 7, 23]. Despite extensive study of proposed mechanisms, it remains unclear why some patients develop insensate versus painful symptoms and how underlying pathological mechanisms determine diabetic neuropathy progression and phenotype.

Rodent Models of Diabetic Neuropathy

Numerous diabetic rodent models have been developed to study diabetic neuropathy. Type 1 diabetes is primarily induced in mice or rats by intra-peritoneal injection of streptozocin (STZ), a selective pancreatic β-cell toxin. Type 2 diabetic models include genetically obese mice due to leptin receptor mutation (*db/db*) or leptin deficiency (*ob/ob*). In addition, type 2 diabetes is commonly modeled in rodents using high-fat diet-induced obesity.

Like human diabetic patients, rodent models display a dichotomous phenotype of diabetic neuropathy. Genetic differences are thought to contribute to neuropathy phenotype in rodents, but the reason why some rodent models develop painful neuropathy while others develop the insensate phenotype remains elusive. Conveniently, rodent models that display variable neuropathy phenotypes allow for investigation into mechanisms that underlie symptom variability.

STZ-induced diabetic rats typically develop mechanical, thermal, and chemical hyperalgesia and reduced sensory and motor nerve conduction velocities, but not insensate symptoms [10, 24]. STZ-induced diabetic mice exhibit a variety of symptoms that vary based on the mouse strain and diabetes duration. Regardless of strain, STZ-diabetic mice typically display

sensory and motor nerve conduction velocity deficits and reduced epidermal innervation [10, 24]; however, STZ-induced C57Bl/6 mice develop mechanical insensitivity while A/J mice develop mechanical hyperalgesia.

Leptin deficient *ob/ob* mice, a model of type 2 diabetes, develop thermal hypoalgesia, mechanical hyperalgesia, and slowed sensory and motor nerve conduction velocities [25-27]. Leptin receptor null mutant mice (*db/db*) also exhibit deficits in sensory and motor nerve conduction velocities [28, 29]. In contrast with the *ob/ob* strain, *db/db* mice develop mechanical insensitivity without changes in thermal sensation [29]. High-fat fed nondiabetic mice that exhibit mechanical hyperalgesia and thermal hypoalgesia are also being utilized to model neuropathy in pre-diabetes [5, 23, 30]. It should be noted that the neuronal cell bodies of the axons that innervate the lower limbs and hind paws reside in the lumbar dorsal root ganglia (DRG). Thus, the lumbar DRG is an important tissue utilized to examine molecular changes in sensory neurons that may affect sensory behavior in rodent models of diabetic neuropathy. Although these models are useful for investigating mechanisms underlying the development of diabetic neuropathy, none of these models fully emulate the human condition.

Dyslipidemia and Lifestyle Factors

Dyslipidemia, typically defined as high serum levels of total cholesterol, low-density lipoprotein cholesterol (LDL-C), high triglycerides, and/or low levels of high-density lipoprotein cholesterol (HDL-C) [31], has recently been identified as a major independent risk factor that increases a diabetic patient's chances of developing neuropathy [5, 7, 23]. For example, the Eurodiab Trial, a longitudinal study of over 3,000 individuals with type 1 diabetes, revealed that

dyslipidemia was independently and closely associated with the risk of developing diabetic neuropathy during a seven year follow-up period [32]. Importantly, dyslipidemia was associated with the onset and progression of neuropathy in both type 1 and type 2 diabetes (reviewed in [33]),[34, 35].

In addition, other lifestyle factors, such as those that comprise the metabolic syndrome, have been associated with neuropathy. The metabolic syndrome is a collection of disorders that when occurring together, increase the risk for developing cardiovascular disease and diabetes [36]. According to the International Diabetes Federation [36], the consensus worldwide definition of the metabolic syndrome is: Central obesity (defined as waist circumference with ethnicity specific values) and two of the following: 1) impaired glucose tolerance (fasting glucose > 100 mg/dl or diabetes), 2) elevated blood pressure (systolic > 130 or diastolic > 85 mm Hg), 3) reduced HDL-C (< 40 mg/dl for women and < 50 mg/dl for men), or 4) elevated triglycerides (> 150 mg/dl) or specific treatment for this lipid abnormality. It is noteworthy to mention that if body mass index (BMI) is > 30 kg/m^2, central obesity can be assumed without waist circumference measurement [36]. Nondiabetic patients with idiopathic neuropathy with and without impaired glucose tolerance had a significantly higher rate of dyslipidemia and hypertension compared to diabetic patients without neuropathy [37]. In addition, the idiopathic neuropathy patients had increased prevalence of the metabolic syndrome compared to several large population based cohorts [37]. BMI has also been independently associated with the incidence of neuropathy in type 1 diabetic subjects [38]. These data suggest that lifestyle factors such as obesity, dyslipidemia, and/or hypertension may influence the development neuropathy in pre-diabetic and diabetic patients. In addition, elevated circulating lipid levels are associated with

insulin resistance and inflammation [39, 40], which have both been suggested as mechanisms that contribute to diabetic neuropathy.

In support of the clinical evidence, nondiabetic rodents fed a high-fat diet exhibit metabolic syndrome parameters including dyslipidemia, hyperinsulinemia, hypertension, elevated glucose, and increased body weight in conjunction with painful neuropathy characterized by mechanical hyperalgesia, thermal hypoalgesia, demyelination, large myelinated and intraepidermal nerve fiber (IENF) loss, and nerve conduction deficits [5, 23, 30, 41]. Vincent et al. demonstrated that non-diabetic C57BL/6 mice exhibited impaired glucose tolerance and developed neuropathy characterized by reduced sensory and motor nerve conduction velocities, sensory deficits, and reduced IENF density after consuming a high-fat diet for 12 weeks [5]. Furthermore, this group showed increased oxidative stress markers including hydroxyoctadecadienoic acid, nitrotyrosine, and dityrosine in plasma of high-fat fed nondiabetic mice [5] compared to mice fed a standard diet.

Although the evidence is strong to suggest that dyslipidemia and other lifestyle factors influence the development of neuropathy, the mechanisms by which this phenomenon occurs are poorly understood. Vincent and colleagues have recently proposed the theory that oxidized low-density lipoproteins (oxLDLs) are one “lipid factor” responsible for nervous system injury in diabetic neuropathy [5, 7]. The oxLDL theory is currently the only proposed mechanism for lipid induced neuronal injury and is described below as summarized by Vincent and colleagues [7]. LDL is the primary lipoprotein that carries cholesterol in the plasma [42]. Importantly, serum oxLDL levels are increased in diabetic patients and are associated with diabetic neuropathy [43, 44] Moreover, oxLDLs are increased in the DRG of high-fat fed mice that exhibit early signs and symptoms of diabetic neuropathy [5].

Oxidative stress can cause modification of lipoproteins and this is well characterized in the atherosclerosis literature [43, 44]. When in the presence of reactive oxygen species (ROS) such as superoxide, LDLs spontaneously oxidize to form oxLDLs [7, 42]). oxLDLs have been shown to cause apoptotic injury and cell death in endothelial cells [45] and neurons [46-48]. Furthermore, oxLDLs increase ROS and activate a caspase-3 dependent cell death mechanism [49] in motoneurons.

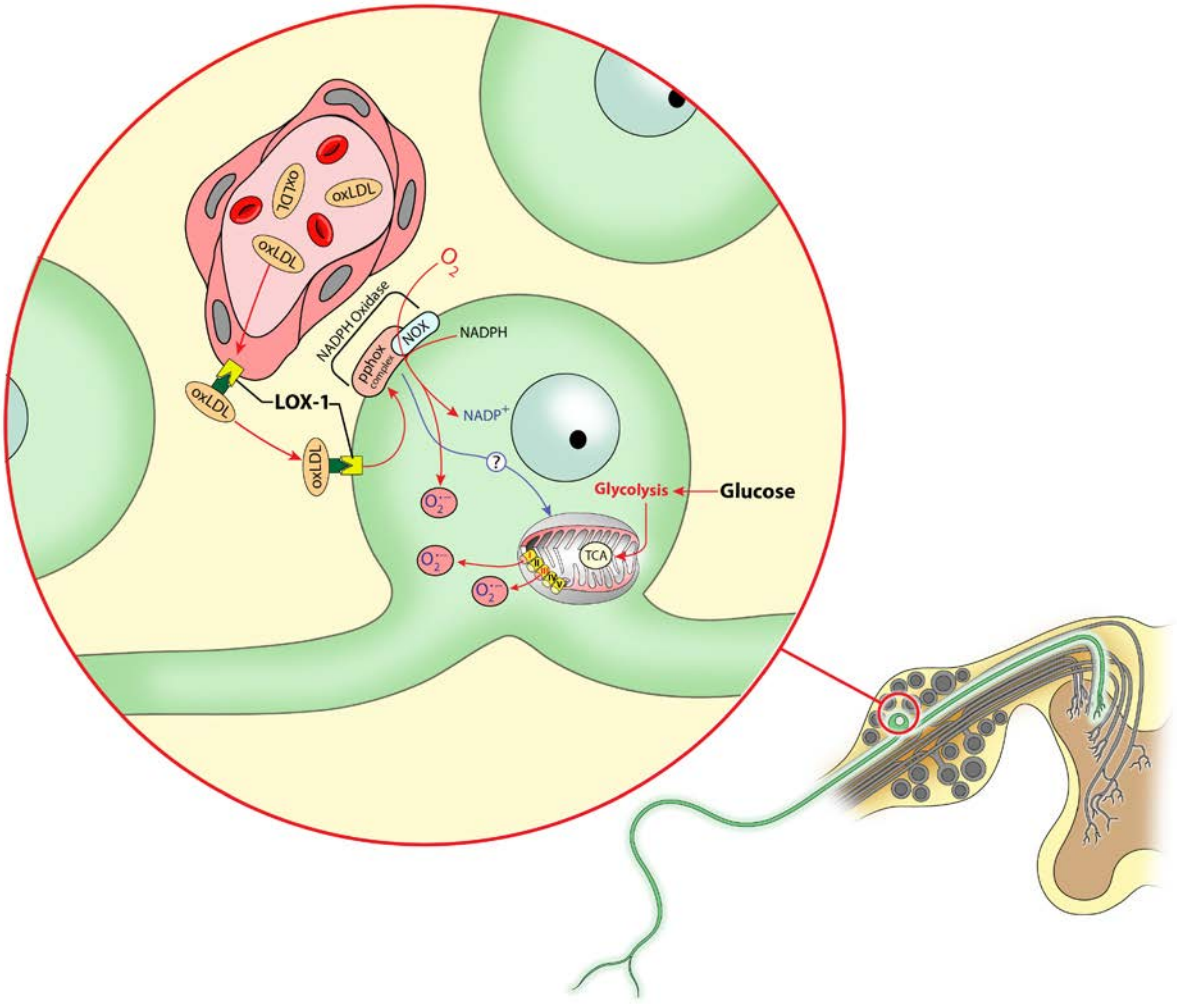
oxLDLs primarily bind to two cell surface receptors including lectin-like oxidized LDL receptor-1 (LOX-1) on endothelial cells [50] and CD36 on macrophages [51]. After binding to its receptor, oxLDL is taken up into the cell [52]. The potential effects of oxLDL binding are illustrated in Figure 1 from Vincent et al. [7] Increased oxLDL levels upregulate LOX-1 expression and consequently cause increased intracellular superoxide production [53]. Interestingly, TNF-α [53], hyperglycemia, and C-reactive protein (CRP) increase LOX-1 expression [54-56], whereas lipid-lowering drugs decrease its expression [57]. Indeed, LOX-1 is expressed on neurons, and LOX-1 gene polymorphisms are associated with neurodegenerative disease in humans [46]. After exposure to oxLDL, LOX-1 expression was increased in cultured DRG neurons from rats [5]. Following oxLDL exposure and LOX-2 activation, NADPH oxidase was subsequently activated (a non-mitochondrial source of superoxide) and consequently, superoxide generation was increased and a programmed cell death mechanism was activated in the DRG neurons. Importantly, neuronal injury in the DRG was prevented by a LOX-1 blocking antibody, apocyanin (NADPH oxidase inhibitor), or the antioxidant α-lipoic acid [5].

Native LDL receptors on neurons play a critical role in neuron function, synapse maintenance, and myelination after injury [58] and oxLDL decreases LDL receptor expression [53]. Therefore, increased oxLDL, via reduced native LDL receptor expression, may contribute

Figure 1. Putative mechanisms for dyslipidemia-induced sensory neuron injury.

Schematic diagram illustrating the DRG, with an enlarged inset of a DRG neuron and adjacent blood vessel. The oxidized form of LDL (oxLDL) binds to the LOX-1 receptor in vascular endothelial cells and DRG neurons and is subsequently endocytosed or transcytosed. oxLDL is thought to activate NADPH oxidase via interactions with the LOX-1 receptor, resulting in non-mitochondrial superoxide generation. In addition, NADPH oxidase may increase mitochondrial production of reactive oxygen species. Finally, glucose may independently induce mitochondrial superoxide production and increase endothelial LOX-1 expression. This schematic illustration was adapted from Vincent et al. [7] with permission from John Wiley and Sons, Ltd.

Figure 1


oxLDL
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NADPH Oxidase
pphox
NOX
NADPH
NADP+
?
Glycolysis
Glucose
TCA

to neuronal injury in individuals with dyslipidemia. These data suggest that dyslipidemia via a LOX-1 dependent mechanism increases ROS generation (independent of hyperglycemia) and induces neuronal damage [7]. Furthermore, when dyslipidemia occurs in the presence of hyperglycemia, neurons may be sensitized to oxLDL-mediated damage due to hyperglycemia-induced upregulation of LOX-1 [7]. These important findings reveal a plausible mechanism to explain the influence of dyslipidemia in the pathophysiology of diabetic neuropathy [5, 7].

Mitochondrial Dysfunction

Mitochondrial dysfunction has been suggested to be another important etiological factor in the multifactorial pathogenesis of diabetic neuropathy. Mitochondria perform several important physiological functions, with energy production being the central and perhaps most vital of these functions. Cells rely on energy produced by mitochondria for all cellular processes including cell growth and maintenance, protein synthesis, and cellular transport. Neurons, due to their relatively high metabolic demands, rely heavily on mitochondrial energy production, which is essential for neuronal growth and survival and maintenance of membrane potential [59]. Neurons often have long processes that require mitochondria to travel long distances from the cell body to the dendritic and axonal termini [60]. Consequently, metabolic disturbances that alter normal mitochondrial function are especially detrimental to neurons, particularly those innervating the distal limbs which are most often affected in diabetic neuropathy [59]. Because neurons are so sensitive to perturbations in mitochondrial function, it is not surprising that mitochondrial dysfunction has been implicated in the pathogenesis of many neurological disorders [61].

Mitochondria participate in numerous physiological functions including fatty acid oxidation, maintenance of calcium homeostasis, cellular apoptosis, oxidative phosphorylation and ATP production. Mitochondria produce ATP by passing electrons down the electron transport chain that is comprised of a series of oxidative phosphorylation complexes located in the mitochondrial inner membrane space. NADH and $FADH_2$ serve as electron donors to the mitochondrial membrane electron transport chain (ETC) enzyme complexes. Electrons are shuttled through these enzyme complexes until they are donated to molecular oxygen, forming water. The electron transfer produces a proton gradient between the inner and outer mitochondrial membrane that drives ATP synthesis [62]. Recent reports indicate mitochondrial dysfunction occurs in peripheral neurons of diabetic animals and suggest these mitochondrial disturbances contribute to development of diabetic neuropathy [63-66]. Studies performed using cultured DRG isolated from diabetic animals reported decreased mitochondrial respiration and maximal respiratory capacity, reduced mitochondrial enzyme complex activity, and decreased expression of mitochondrial oxidative phosphorylation proteins, compared to nondiabetic animals [63, 64, 66]. In addition, cultured DRG neurons from diabetic animals exhibit aberrant mitochondrial trafficking, mitochondrial accumulation in axon swellings, and altered mitochondrial fission-fusion equilibrium resulting in small mitochondria with ultrastructural abnormalities [64, 67-70]. Furthermore, changes in mitochondrial number and size have been reported in the Schwann cells of myelinated and unmyelinated axons during peripheral nerve regeneration in diabetic humans and animals [71].

To date, all published studies of mitochondrial dysfunction in diabetic rodents have been performed in primary DRG cultures, raising the question of whether mitochondrial dysfunction drives the development of neuropathy or if it is merely a consequence of neuronal damage [64,

67-70]. When studied in culture, neurons are in a regenerative state of repair, thus observations of mitochondrial dysfunction in cultured DRG neurons may suggest that mitochondrial dysfunction plays a role in impaired nerve regeneration that occurs downstream of dying-back axonal degeneration in diabetic neuropathy.

The Role of Oxidative Stress in Mitochondrial Dysfunction

The term oxidative stress refers to the excess production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) [18]. As electrons are passed from oxidative phosphorylation complex II to III in the electron transport chain, ROS are produced as byproducts. In normal metabolism, ROS production is minimal and antioxidant enzymes including superoxide dismutase, catalase, and glutathione function to detoxify the mitochondria of ROS [18, 62]. Hyperglycemia causes increased glucose metabolism that generates an overabundance of NADH and $FADH_2$ electron donors. This produces a high proton gradient across the inner mitochondrial membrane, which increases the turnover of the enzyme complexes, and results in increased levels of ROS [18, 62]. Subsequently, ROS production exceeds the detoxifying capability of the mitochondrial antioxidant enzymes. Oxidative stress can also activate the p-ADP ribose (PARP) pathway [72], which in conjunction with the hexosamine and protein kinase C (PKC) pathways, induces inflammation and neuronal dysfunction [3]. In addition, oxidative stress in combination with hyperglycemia activates advanced glycation end product (AGE), polyol, hexosamine, and PKC pathways which lead to further oxidative stress, cellular redox imbalance, and gene expression disturbances [3]. Activation of these detrimental pathways can also induce inflammation and neuronal dysfunction

[3]. Since excess ROS results in activation of these detrimental pathways that produce additional ROS, oxidative stress becomes a cyclical problem.

Despite the fact that mitochondria are a source of ROS, they are often the first structures to be damaged, thus putting the neuron at greater risk [18]. ROS accumulation can be very detrimental to mitochondrial DNA, mitochondrial membranes, and the whole cell [3, 62]. Diabetic mice have decreased levels of superoxide dismutase suggesting reduced capacity for ROS detoxification [67]. However, decreased mitochondrial respiration was not associated with increased ROS production in these studies [66, 67]. Taken together, these data suggest that diabetes-induced mitochondrial dysfunction does not necessarily result in increased mitochondrial ROS generation [66]. Mitochondrial dysfunction has been identified an important etiological factor in the pathogenesis of diabetic neuropathy, but the contributions of mitochondrial dysfunction in diabetic neuropathy remain controversial [20, 22, 66, 73].

Inflammation

Inflammatory mechanisms play an important role in the pathogenesis of both type 1 and type 2 diabetes and have recently been implicated as key mediators in the progression of diabetic complications including nephropathy, retinopathy, and neuropathy [74]. Diabetes is typically accompanied by a chronic state of whole body low-grade inflammation that can be exacerbated by hyperglycemic fluctuations [75]. Characteristic of chronic inflammation, pro-inflammatory cytokines including tumor necrosis factor alpha (TNF-α), C-reactive protein (CRP), interleukin (IL)-6, and IL-1β are elevated in serum from diabetic patients [76, 77]. Adipose tissue is a key

source of pro-inflammatory cytokines and inflammatory markers are also increased in adipose from diabetic humans and rodents [77, 78].

Inflammation has been identified as a mechanism contributing to insulin resistance [76], thus this line of research has more often focused on the role of inflammation in type 2 diabetes rather than type 1 diabetes. Obesity, a common concomitant condition with type 2 diabetes, can also induce inflammation and is associated with chronic low-grade inflammation [79-82]. It is also purported that diet-induced hyperlipidemia may contribute to inflammation and diabetic complications [40, 83]. Although the role of chronic inflammation in the pathogenesis of type 1 diabetes is not well understood, increased pro-inflammatory cytokines and chemokines have been documented in type 1 diabetic patients [84, 85]. It has been suggested that inflammation plays a larger role in the long-term progression of type 1 diabetes rather than its onset [86]. Although inflammation may play slightly different roles in the progression of type 1 and type 2 diabetes, it is clear that the inflammatory process is linked with the development of diabetic complications [74, 86]. Hyperglycemia, a feature common to both type 1 and type 2 diabetes, acts deleteriously on a number of pathways including the aldose reduction, AGE, reactive oxygen intermediate, and protein kinase C pathways [86]. All of these pathways can induce the production of inflammatory mediators that, in turn, activate innate immune cells, which can damage the pancreas, adipose and vasculature, thus leading to further diabetic complications [76]. Although this pro-inflammatory process is essential for tissue damage and repair, chronic activation can lead to pathological changes and result in disease [87].

Numerous studies have documented increased inflammatory mediators in serum and tissues of humans and rodent models of diabetes and in many cases, these increases were specifically linked to diabetic neuropathy (reviewed in [88]). Of the cytokines, many of the

interleukins and TNF-α have been implicated in neuropathic conditions. Nondiabetic patients with small-fiber neuropathy show increased gene expression of IL-2 in peripheral blood and increased Il-6 and IL-8 in skin biopsies compared to control subjects [89]. Importantly, further study revealed that these patients had robust increases in IL-1β, Il-6, and Il-8 mRNA in skin affected by spontaneous pain compared to unaffected skin [89]. Sural nerve biopsies from patients with diabetic neuropathy also reveal increased Il-6 protein expression compared to healthy controls [90]. In addition, STZ-induced diabetic mice with neuropathy have increased IL-6 gene expression in the DRG and sciatic nerve compared to nondiabetic mice [89, 90].

TNF-α is a classic pro-inflammatory cytokine that can induce nociceptive behavior in rodents [91] and ectopic firing [92] in sensory neurons, and has been implicated in neuropathic and inflammatory nociceptive conditions in numerous studies over several years [88]. Although, TNF-α has only recently begun to be studied in diabetic neuropathy, TNF-α has quickly become the inflammatory mediator with the strongest evidence linking diabetic neuropathy and inflammation [88]. Diabetic patients and STZ-diabetic rodents have increased TNF-α plasma protein and mRNA levels compared to nondiabetic controls [93-98]. To substantiate the importance of TNF-α in the development of diabetic neuropathy, TNF-α null mutant diabetic mice fail to develop the nocifensive behavior changes and sensory and motor nerve conduction deficits that are normally observed in diabetic wild-type mice [99]. Furthermore, treatment with a TNF-α neutralizing antibody ameliorated diabetic neuropathy in STZ-induced diabetic mice [99].

Although greater focus has been placed on the role of cytokines in diabetic neuropathy, increased levels of other inflammatory mediators including chemokines, NF-κβ, toll-like receptors (TLRs) [100], and cyclooxygenase-2 (Cox-2) [101-103] have also been documented in

diabetic rodents and/or humans with neuropathy. NF-κβ is a transcription factor that induces several inflammatory and immune mediators and increased NF-κβ activation has been reported in several tissues in diabetic patients and in the DRG, sural, and sciatic nerve of diabetic mice [90, 104, 105]. Activated TLRs induce NF-κβ and subsequently increase cytokine and chemokine expression [88]. Type I diabetic patients exhibit increased circulating levels of TLR ligands and increased TLR2 and TLR4 monocyte surface expression [100]. Clinical studies indicate diabetic patients have increased Regulated on Activation Normally T-cell Expressed and Secreted (RANTES) and Monocyte Chemoattractant Protein-1 (MCP-1) levels compared to healthy control subjects [106, 107]. Furthermore, diabetic patients with neuropathy had increased circulating RANTES levels compared to diabetic patients without neuropathy while increased serum MCP-1 was positively correlated with peripheral neuropathy in diabetic patients [106, 107].

It has been suggested that cytokine levels may play a role in dictating whether a patient experiences a painful or painless phenotype of neuropathy. In fact, clinical studies have indicated that patients (not necessarily diabetic) with painful neuropathy have increased serum TNF-α and IL-2 compared to patients with painless neuropathy [95]. In addition, patients with painless neuropathy had increased IL-10, an anti-inflammatory cytokine, compared to patients with painful neuropathy [95]. In support of the clinical data, STZ-induced diabetic rats with hypoalgesia have decreased protein levels of TNF-α in the DRG [108] while STZ-diabetic rats that exhibit hyperalgesia display increased TNF-α levels in the DRG [99]. Taken together, these data make a strong case for the role of inflammatory mediators in the pathogenesis of diabetic neuropathy, with specific emphasis on the painful neuropathy phenotype.

Peripheral Nervous System Insulin Resistance

Insulin resistance has been well established as a central feature of type 2 diabetes and is defined as reduced target tissue responsiveness to normal levels of circulating insulin [109]. While insulin deficiency is the primary factor in the pathogenesis of type 1 diabetes, patients with type 1 diabetes can also develop insulin resistance [110, 111]. In fact, recent evidence suggests insulin resistance contributes to micro- and macro- vascular complications in type 1 diabetes [110-113]. In type 2 diabetes, insulin resistance is the key feature of this disease and the primary cause of hyperglycemia. Insulin resistance in the setting of diabetes has been thoroughly characterized in insulin-dependent tissues including muscle, fat, and liver [114-116]. Although neurons are not insulin-dependent, they are insulin-responsive, and emerging evidence now demonstrates that insulin resistance also occurs in neurons [117, 118]. Importantly, reduced neuronal insulin signaling (due to insulin deficiency in type 1 or insulin resistance in type 2 diabetes), has recently been proposed as a mechanism contributing to neurodegeneration and the pathogenesis underlying diabetic neuropathy [117, 119].

Insulin is an essential hormone required for maintaining glucose homeostasis via regulation of hepatic gluconeogenesis and glucose uptake by muscle and liver. Insulin is critical for glucose uptake in muscle and adipose, but neuronal glucose uptake occurs in an insulin-independent manner [120]. Although neurons are not dependent on insulin for glucose update, neurons rely on insulin as a key neurotrophic factor important for neuronal growth [121, 122], plasticity, regeneration [123-125], and maintenance of mitochondrial function [21, 66].

Insulin signaling occurs in a series of phosphorylation events following insulin binding to its extracellular receptor [126]. Following insulin binding, the insulin receptor autophosphorylates, becomes activated, and subsequently phosphorylates several intracellular

substrates including members of the insulin receptor substrate (IRS) family on their tyrosine residues [127]. Tyrosine phosphorylation on IRS allows downstream signaling molecules containing Src homology 2 domains (SH2) to bind IRS, then localize to the plasma membrane thereby becoming activated [128]. Growth factor receptor-binding protein-2 (Grb-2) and PI-3 kinase are two of the key SH-2 containing mediators that undergo activation by IRS [128, 129]. Insulin signals through two different pathways: PI-3 kinase activates the Akt cascade while Grb-2 activates the mitogen-activated protein kinase (MAPK) branch of insulin signaling [109]. In insulin dependent tissues via the Akt cascade, insulin stimulates glucose uptake by inducing Glut4 translocation to the plasma membrane, glycogen synthesis, protein synthesis via mTor activation, fatty acid synthesis, promotes cell survival, and inhibits lipolysis. In addition, insulin signaling stimulates cell growth and mitogenesis through activation of the MAPK cascade [109].

A growing body of evidence documenting impaired neuronal insulin signaling in animal models of diabetic neuropathy supports the idea that neuronal insulin resistance is an important mechanism contributing to the pathogenesis of diabetic neuropathy. Metabolic disturbances including obesity, hyperglycemia, hyperinsulinemia, and inflammation can cause disturbances in the insulin signaling pathway [109]. In pathological conditions, serine phosphorylation on IRS replaces the normal IRS tyrosine phosphorylation thus inhibiting normal insulin signal transduction and downstream effects [128-131]. In fact, increased serine phosphorylation on IRS2, the prevalent neuronal IRS isoform, has been reported in DRG neurons from type 1 and type 2 diabetic mice [132]. Other derangements in the insulin-signaling pathway have been reported in the DRG from *ob/ob* mice including reduced insulin-stimulated Akt activation and impaired neurite outgrowth [117, 132]. In addition, low-dose intrathecal, but not subcutaneous, insulin injection improved deficits in sensory and motor nerve conduction velocities in type 1

diabetic rats without affecting hyperglycemia [119]. Low-dose insulin administration also relieved mechanical hyperalgesia in type 1 diabetic rats despite persistent hyperglycemia [133]. Furthermore, STZ-diabetic rats with moderate insulinopenia without hyperglycemia exhibit symptoms of neuropathy [134], suggesting loss of insulin signaling, apart from insulin's effects on glycemia, may be a driving factor behind neuronal damage and degeneration in diabetic neuropathy. Taken together, these data suggest that impaired neuronal insulin signaling and neurotrophic support, whether due to insulin deficiency or insulin resistance, play an important role in the pathophysiology of diabetic neuropathy.

Exercise Intervention as a Preventative and Treatment for Diabetic Neuropathy

No definitive cure exists for diabetic neuropathy and current pharmacological therapeutics have shown limited success in treating the symptoms of this debilitating disease. Antidepressants, antiepileptics, and opioids are commonly used to treat neuropathic pain, yet have. limited effectiveness, significant side effects, no impact on hyperglycemia-induced cell damage, and no affect on nerve regeneration [135, 136]. Although only a few studies have been reported,, exercise has been shown to have both protective [137, 138] and therapeutic effects [138, 139] on diabetic neuropathy. Exercise affects the multifactorial pathogenesis underlying diabetic neuropathy by targeting numerous pathophysiological mechanisms all at once. In addition, exercise can potentially reverse nerve fiber damage, and induce neurogenesis and synaptic plasticity [140]. Exercise is safe, effective, and capable of affecting numerous targets with limited side effects, and these characteristics are nearly impossible to achieve in single or even multiple pharmacotherapeutics.

Numerous reports document the beneficial effects of exercise on mitochondrial dysfunction [141-144], oxidative stress [145], dyslipidemia [146, 147] , insulin resistance [148, 149] inflammation [150, 151], and neurotrophic support [152]. Aerobic exercise training is well-known for its ability to improve mitochondrial function and induce mitochondrial biogenesis in skeletal muscle and these effects have been frequently studied in both diabetic patients and rodents [141-144]. In addition, exercise can increase axonal regeneration through a neurotrophin dependent mechanism [152]. Markers of oxidative stress are also diminished after chronic exercise training in diabetic patients and diabetic rodents [145, 153]. Additionally, aerobic exercise training significantly improves dyslipidemia in nondiabetic and diabetic humans [146, 154]. Exercise is especially beneficial for improving insulin sensitivity in muscle, liver, and/or adipose of diabetic patients and rodents [150, 155, 156]. Furthermore, exercise reduces the level of pro-inflammatory cytokines in adipose [150, 153, 157-159], serum [151, 160-164], and liver [165]. In fact, recent evidence demonstrates that exercise pre-conditioning prevents brain inflammation following stroke suggesting that exercise can modulate inflammation in neural tissue [166]. Although this large body of evidence reports exercise-induced improvements in several mechanisms that are purported to contribute to diabetic neuropathy in the peripheral nervous system, these beneficial effects were observed in other peripheral tissues. Further investigation is needed to elucidate the effects of exercise on inflammation, insulin sensitivity, oxidative stress, and mitochondrial function in neural tissues, thus shedding light on potential mechanisms underlying the beneficial effects of exercise on diabetic neuropathy.

Although little research has been done on the effects of exercise on diabetic neuropathy, the few studies that have been done indicate exercise can improve and maintain nerve function in patients with diabetic neuropathy [137, 139, 167, 168]. In a relatively large longitudinal study,

type 1 and type 2 diabetic patients without neuropathy who performed brisk treadmill walking for 4 hrs/wk for four years had less incidence of motor and sensory neuropathy than control sedentary diabetic patients [137]. In addition, sensory and motor nerve conduction velocities were higher in the exercise group and the percentage of diabetic patients who developed increased vibration perception threshold was higher in the control group, suggesting exercise prevented deleterious sensorimotor changes [137]. Following a 24-week aerobic exercise program, sensory and motor nerve conduction velocities were improved in type 2 diabetic patients with diabetic neuropathy [139]. Moderate aerobic exercise training has also been shown to reduce pain and increase epidermal nerve fiber density [168]. Kluding and colleagues executed one of the few human studies that incorporated both aerobic and strength training exercise in diabetic patients with neuropathy [169]. After 10 weeks of 3-4 days per week of exercise training, diabetic patients with neuropathy had a 30% decline in pain severity, improved neuropathy severity score, and increased epidermal nerve fiber branching [169]. These results strongly support the use of exercise as a powerful and effective therapeutic for preventing and reversing symptoms of diabetic neuropathy in human patients.

Exercise studies in rodent models of diabetic neuropathy further corroborate the clinical evidence. Treadmill exercise delays the development of thermal and mechanical hyperalgesia in STZ-induced diabetic rats compared to sedentary STZ-diabetic rats [170]. In addition, swimming exercise prevented motor dysfunction in STZ-induced diabetic rats [138], thus bolstering the case for the protective and therapeutic effects of exercise on diabetic neuropathy. Despite the lack of understanding surrounding the mechanisms underlying the beneficial effects of exercise on diabetic neuropathy, the evidence strongly points to exercise as a promising preventative and treatment intervention for this disease.

Study Significance

Diabetic neuropathy is a debilitating disease that places a significant physical and emotional burden on patients who suffer from the unrelenting symptoms. Unfortunately, current treatments are inadequate and show limited success at treating the symptoms of this disease. Although the majority of diabetic patients will develop some form of peripheral neuropathy, it is unknown why some diabetic patients escape neuropathic symptoms. Additionally, it is unclear why diabetic neuropathy presents with a dichotomous phenotype such that some patients present with painful neuropathy while others experience painless symptoms including loss of sensation.

Recently, dyslipidemia has been identified as an independent risk factor for the development of neuropathy in diabetic patients [7]. It has also been demonstrated that a high-fat diet induces neuropathy in pre-diabetic rodents [5, 23, 41]. When the clinical and basic evidence is considered together, it is plausible to suggest that diet plays an important role in the development of neuropathy and may have an effect on neuropathy phenotype. The overall goal of this body of work is to better understand how lifestyle factors such as diet may predispose a diabetic patient to develop neuropathy in an effort to supplement the available pharmaceutical treatments with additional clinical guidance. In particular, these studies were aimed at providing mechanistic evidence to justify the use of novel therapeutic interventions such as dietary modification and exercise to prevent and treat diabetic neuropathy.

The goal of the first study **("Phenotypic Changes in Diabetic Neuropathy Induced by a High-Fat Diet in Diabetic C57Bl/6 Mice")** was to characterize the effects of a high-fat diet on neuropathy development and progression and in nondiabetic and STZ-induced diabetic mice. We discovered that a high-fat diet induced mechanical hyperalgesia in both nondiabetic and diabetic mice. Considering that STZ-induced diabetic mice normally exhibit insensate neuropathy, the

observation that high-fat feeding reverses this phenotype is a transformational finding in the diabetic neuropathy field. The three studies that followed were designed to elucidate the potential mechanisms underlying high-fat diet-induced mechanical hyperalgesia.

In the second study, **"Mitochondrial Function in Diabetic Neuropathy"**, we examined the effects of high-fat feeding on mitochondrial respiration and expression of proteins involved in oxidative phosphorylation or mitochondrial function in DRG of nondiabetic and STZ diabetic mice. Our findings demonstrated that basal mitochondrial respiration was reduced while mitochondrial proteins were elevated in STZ-diabetic mice fed a standard diet. These results indicate that diabetes, but not the high-fat diet, induced perturbations in mitochondrial function.

In the third study, **"The Role of Inflammation in High-Fat Diet-Induced Hyperalgesia"**, we investigated the effects of high-fat feeding on spinal and peripheral inflammation along with microglial activation in nondiabetic and STZ-diabetic mice. Our results indicated that high-fat feeding did not increase spinal or peripheral inflammation or induce microglial activation in nondiabetic or diabetic mice.

In the final study, **"The Effects of Exercise on Insulin Signaling in Mice Fed a High-Fat Diet"**, we evaluated the effects of exercise on 1) serum glucose and insulin levels and 2) insulin signaling in the DRG, sciatic nerve, and gastrocnemius, in high-fat mice. Diabetic mice were not used in this study, as it was designed to determine if exercise could ameliorate high-fat feeding-induced impairments in neuronal insulin sensitivity. Our findings demonstrated that exercise prevented increased body weight and circulating glucose and insulin levels, normally observed in high-fat fed mice. In addition, exercise prevented high-fat diet-induced derangements in insulin signaling in the gastrocnemius. The high-fat diet did not result in statistically significant impairments in insulin signaling in the DRG or sciatic nerve, nor was

insulin signaling in exercised high-fat fed animals were not different from sedentary animals. This study reinforced the importance of exercise in preventing or reversing symptoms of diabetes and advocates for the use of exercise to prevent or treat complications associated with diabetes.

CHAPTER 2

Phenotypic Changes in Diabetic Neuropathy Induced by a High-Fat Diet in Diabetic C57Bl/6 Mice

1. Abstract

Emerging evidence suggests that dyslipidemia is an independent risk factor for diabetic neuropathy (reviewed in [7]). To experimentally determine how dyslipidemia alters diabetic neuropathy, we quantified neuropathic symptoms in diabetic mice fed a high-fat diet. Streptozocin (STZ)-induced diabetic C57BL/6 mice fed a high-fat diet developed dyslipidemia and a painful neuropathy (mechanical hyperalgesia) instead of the insensate neuropathy (mechanical insensitivity) that normally develops in this strain. Nondiabetic mice fed a high-fat diet also developed dyslipidemia and mechanical hyperalgesia. Thermal sensitivity was significantly reduced in diabetic compared to nondiabetic mice, but was not worsened by the high-fat diet. Moreover, diabetic mice fed a high-fat diet had significantly slower sensory and motor nerve conduction velocities compared to nondiabetic mice. Overall, dyslipidemia resulting from a high-fat diet may modify diabetic neuropathy phenotypes and/or increase risk for developing diabetic neuropathy. These results provide new insight as to how dyslipidemia may alter the development and phenotype of diabetic neuropathy.

2. Introduction

The prevalence of societal overweight, obesity, and physical inactivity continue to increase, thus the influence of lifestyle-related metabolic variables has become increasingly important in terms of diabetic neuropathy risk and progression. Data from several large clinical trials suggest that dyslipidemia, typically defined as high serum levels of total cholesterol, low-density lipoprotein cholesterol (LDL-C), high triglycerides, and/or low levels of high-density lipoprotein cholesterol (HDL-C) [31], is a major independent risk factor for the development of diabetic neuropathy (reviewed in [7]). In addition, dyslipidemia is associated with the onset and progression of neuropathy in both type 1 and type 2 diabetes (reviewed in [33]),[34, 35]. Body mass index has also been independently associated with the incidence of neuropathy in type 1 diabetic subjects [38]. Most individuals with neuropathy associated with pre-diabetes have painful small-fiber sensory neuropathy, are obese, and have dyslipidemia [11, 171-174]. Moreover, results from a cross-sectional study of type 2 diabetic subjects revealed that the prevalence of diabetic neuropathy was two-fold higher in type 2 diabetic subjects with the metabolic syndrome [175], a condition characterized by dyslipidemia, obesity, and hyperglycemia [176]. In support of the clinical evidence, nondiabetic mice fed a high-fat diet exhibit dyslipidemia [5], increased body weight, and painful neuropathy characterized by mechanical hyperalgesia, thermal hypoalgesia, and nerve conduction deficits [5, 23].

Although diet was not assessed in the clinical studies, it is plausible to suggest that diet may indirectly affect diabetic neuropathy progression and phenotype since dyslipidemia and obesity in adult humans can often be attributed to excess energy and fat intake. Taken together, this evidence suggests that diet may modulate the progression and phenotype of diabetic neuropathy, and these effects may be mediated in part by diet-induced dyslipidemia and/or

excess body weight. Although neuropathy and pre-diabetes has been documented in C57Bl/6 mice fed a high-fat diet [5, 23], the effects of a high-fat diet in conjunction with type 1 diabetes has not been studied. Here, we report the effects of a high-fat diet on neuropathy progression and phenotype in streptozotocin (STZ)-induced type 1 diabetic mice.

3. Experimental Procedures

Animals and Diet

Seven week-old male C57Bl/6 mice were purchased from Charles River (Wilmington, MA), housed two mice per cage under pathogen free conditions, and placed on a 12:12h light/dark cycle in the research support facility at the University of Kansas Medical Center. All animals had ad libitum access to food and water and were fed a standard diet (8604; Harlan Teklad, Madison Wisconsin; 14% kcals from fat, 32% protein, and 54% carbohydrate) or high-fat diet (07011; Harlan Teklad; 54% kcals from fat comprised of lard and corn oil, 21% protein, and 24% carbohydrate). Animals in the high-fat diet group were fed the standard diet for 1 week prior to STZ-injection and began the high-fat diet 3 hours post STZ-injection. All protocols and procedures were approved by the University of Kansas Medical Center Animal Use and Care Committee.

Diabetes Induction

A single intraperitoneal STZ injection (180 mg/kg body weight; Sigma-Aldrich, St. Louis, MO) in 10 mM sodium citrate buffer (pH 4.5) was administered to 8 week-old C57Bl/6 mice to induce diabetes. Eight week-old nondiabetic C57Bl/6 mice were injected with 400µl

vehicle (sodium citrate) buffer. All mice were fasted for 3 hours pre and post STZ-injection. Body weight and blood glucose (glucose diagnostic reagents, Sigma, St. Louis, MO) were monitored 1 week post-STZ injection and every week thereafter. Mice with blood glucose levels of > 300 mg/dl (> 16 mmol/L) were considered diabetic. STZ-injected mice with blood glucose levels below 300 mg/dl were excluded from the study. Treatment groups are abbreviated throughout as follows: nondiabetic standard diet (NdStd); nondiabetic high-fat diet (NdHF); diabetic standard diet (DbStd); and diabetic high-fat diet (DbHF).

Behavioral Testing

Behavioral testing to assess characteristic signs of diabetic neuropathy including mechanical sensitivity, thermal sensitivity, and sensorimotor ability (beam walk task) were performed 1 week before STZ-injection and subsequently thereafter. Before each behavioral test was performed for the first time, mice were acclimated to the apparatus during two separate training sessions. Immediately prior to each behavioral test, mice were acclimated to the behavior room for 30 minutes followed by a 30-minute acclimation on the testing apparatus (except beam walk).

Mechanical Sensitivity. Mice were placed on an elevated wire mesh screen (55 cm above table), enclosed individually in clear plastic cages (11 x 5 x 3.5 cm), and mechanical sensitivity was assessed using a 1.4 g von Frey monofilament (Stoelting, Wood Dale, IL) which was applied 6 times to each hind paw footpad. A combined mean percent withdrawal from a total of 12 applications was calculated per mouse and used to calculate group means.

Thermal Sensitivity. Mice were placed on a Hargreaves apparatus and 4.0 V radiant heat source was applied four times to each hind paw footpad. Time elapsed before the animal withdrew the paw was recorded as withdrawal latency. A combined mean withdrawal latency (secs) was calculated from a total of 8 applications per mouse and used to calculate group means.

Beam Walk. Mice were trained to traverse a 1 m-long, 1.2 cm diameter, wooden beam (adapted from [177, 178]). As each animal crossed the beam, the number of times the right or left paw slipped off the beam was counted as a footslip. This behavioral test was recorded using a digital video camera. The combined mean number of footslips/mouse was calculated from a total of 3 trials per session and used to calculate group means.

Nerve Conduction Velocity

At 8 weeks post-STZ and immediately before sacrifice, mice were deeply anesthetized by intraperitoneal injection with Avertin (1.25% v/v tribromoethanol [Sigma-Aldrich] c, 2.5% tert-amyl alcohol [Sigma-Aldrich], dH_2O; 200 μL/10 g body weight) and motor and sensory nerve conduction velocities were recorded according to Stevens et al. [179] and as described previously by Muller et al. [180]. Body temperature was monitored by rectal probe and maintained at 37 ^{0}C.

Serum Lipids and Insulin

At 8 weeks post-STZ injection and immediately following NCV studies, anesthetized mice were sacrificed. Blood was collected into Eppendorf tubes, placed on ice to clot for 30 minutes, and centrifuged for 15 minutes at 3,000 x *g*. Serum was removed and frozen at -80^{o}C. Serum samples were assayed for total cholesterol (Cholesterol Total E kit, Wako Diagnostics)

LDL-C (L-type LDL-C kit, Wako Diagnostics), triglycerides (triglyceride kit, Cayman Chemical), and insulin (mouse insulin Elisa, Alpco).

Immunohistochemistry

Anesthetized mice were sacrificed and tissues were dissected at 8 weeks post-STZ injection. Unfixed lumbar dorsal root ganglia (DRG) were dissected and frozen in Tissue-Tek O.C.T. Compound (OCT, Sakura, Torrance, CA). Hind paw footpads were dissected and fixed for 2 hours in Zamboni's fixative (4% formaldehyde, 14% saturated picric acid, 0.1 M phosphate buffered saline [PBS, pH 7.4] at 4°C), immersed overnight in 1% PBS (pH 7.4 at 4°C), and finally immersed for 4 hours in 30% sucrose in 1X PBS (pH 7.4 at 4°C). After freezing in OCT, DRG and footpads were sectioned on a cryostat at 10μm and 30μm, respectively, mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA), and stored at. -20°C.

After thawing for 5 minutes at room temperature, slide mounted tissue was covered with blocking solution (0.5% porcine gelatin, 1.5% normal donkey serum, and 0.5% Triton-X in Superblock buffer; Pierce) for 1 hour at room temperature. Slides were then incubated overnight at 4° C in primary antibody diluted in blocking solution. Slides were washed the following day for 2 x 10 min with PBST followed by 3-hour incubation with fluorochrome-conjugated secondary antibodies diluted in PBS and blocking solution. Following 2 x 10 min washes in 1X PBS, slides were rinsed in deionized distilled H_2O, coverslipped and stored at 4 ^{0}C (footpad sections) or cover slipped with Anti-Fade Prolong Gold (Invitrogen, Carlsbad, CA) and stored at room temperature (DRG sections).

Intraepidermal Nerve Fiber Density

Rabbit anti-PGP 9.5 (1:400; Chemicon, Temecula, CA) and Alexa-488 (1:2000; Molecular Probes, Eugene, OR) were used to label and visualize dermal and epidermal nerve fibers. Fluorescent digital images were acquired from epidermal regions using a Nikon Eclipse E800 microscope. The number of nerve fibers per section that cross the epidermal-dermal border were counted using a 40x objective in order to visualize fibers throughout the full depth of the tissue section. NIH Image J software was used to measure each epidermal region and intraepidermal nerve fiber density (IENFD) was expressed as number of fibers per millimeter of epidermis. The combined mean IENFD from 6 images per mouse was used to calculate group means.

Oxidative Stress

Primary antibodies (rabbit anti-nitrotyrosine [1:1000; Chemicon, Temecula, CA] and rabbit anti-neurofilament H [1:10,000, Chemicon, Temecula, CA]) and fluorescent secondary antibodies (Alexa 488 and Alexa 555 [both 1:2000; Molecular Probes, Eugene, OR]ere used to label and visualize nitrated tyrosine residues and neurons, respectively, in the lumbar DRG. Six fluorescent digital images from 6 DRG sections per mouse were acquired at 40x using a Nikon Eclipse E80i microscope. All pictures were taken using the identical exposure time. Metamorph software (MDS Analytical Technologies, Downington, PA) was used to quantify the mean intensity of nitrotyrosine labeling in each image (neuron cell body specific nitrotyrosine fluorescence intensity minus background intensity). Compound mean fluorescence intensity from 6 images per mouse was used to calculate group means. In addition, neuron cell body-specific

nitrotyrosine fluorescence intensity was expressed as a percentage of total regions per mouse and binned as: low (≤ 300 intensity units), medium (301 – 900 intensity units), or high (> 900 intensity units), and used to calculate group means.

Statistics

Data were analyzed using a two-factor analysis of variance (ANOVA) or repeated measures ANOVA with Fisher's test of least square difference post-hoc comparisons. Statistical significance was set at $P < 0.05$.

4. Results and Figures

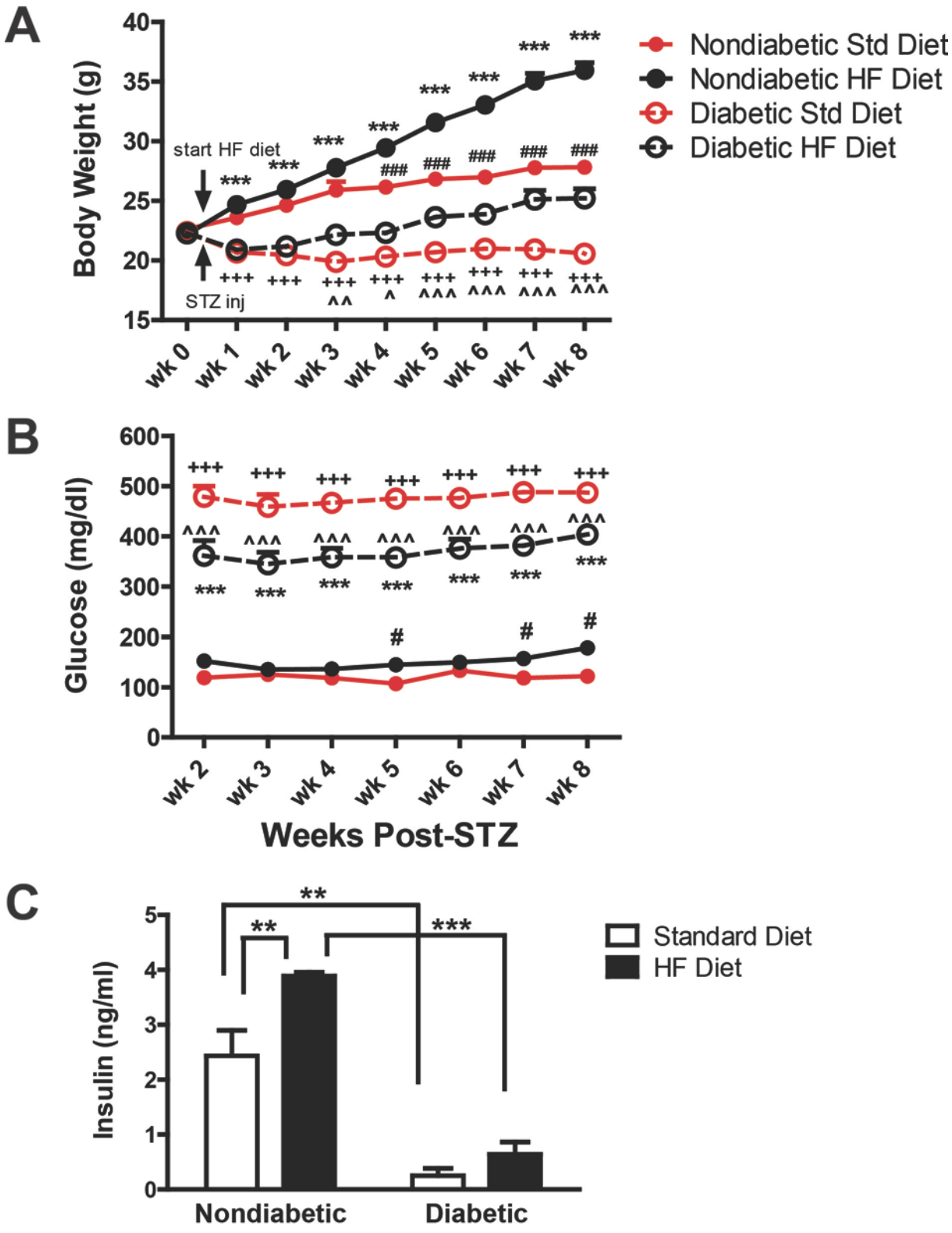
Body Weight, Glucose, and Insulin

Diabetic mice displayed common symptoms of diabetes, including polydipsia and polyuria within 1 week after STZ-injection. Typical with this model, diabetic mice weighed less than their nondiabetic counterparts. However, high-fat fed diabetic mice gained weight (+3 g) compared to diabetics fed the standard diet who lost 1.7 g on average (Figure 1A). Nondiabetic mice on the standard diet gradually gained 5 g in body weight, whereas high-fat fed nondiabetic mice gained 13 g over the course of the 8-week study (Figure 1A). Energy intake was not different among nondiabetic groups (NdStd 13.5 ± 1.3 kcals/day; NdHF 14.0 ± 0.5 kcals/day, $P > 0.05$ for NdStd vs. NdHF). In contrast, diabetic mice on the standard diet consumed at least 2 times as many kcals/day compared to all other groups (DbStd 29.7 ± 1.8 kcals/day; DbHF 15.2 ± 7.8 kcals/day, $P < 0.05$ for DbStd vs. NdStd, NdHF, and DbHF).

Figure 1: The effects of STZ-induced diabetes and high-fat feeding on body weight fasting blood glucose.

Both diabetic groups display characteristic signs of diabetes including lower body weight and severe hyperglycemia compared to nondiabetic mice. High-fat fed mice gain more weight and have lower glucose levels than their standard diet counterparts. Body weight (A) and blood glucose (B) (n = 14-21 mice per group). $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ for NdStd vs. NdHF; $^{+++}P < 0.001$ for NdStd vs. DbStd; $***P < 0.001$ for NdHF vs. DbHF; $^P < 0.05$, $^^P < 0.01$, and $^^^P < 0.001$ for DbStd vs. DbHF. C) Serum insulin (n = 3 – 8 mice per group). $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Data are presented as means ± SEM.

Figure 1


A
Body Weight (g)
start HF diet
STZ inj
Nondiabetic Std Diet
Nondiabetic HF Diet
Diabetic Std Diet
Diabetic HF Diet
wk 0
wk 1
wk 2
wk 3
wk 4
wk 5
wk 6
wk 7
wk 8
B
Glucose (mg/dl)
Weeks Post-STZ
C
Insulin (ng/ml)
Standard Diet
HF Diet
Nondiabetic
Diabetic

In nondiabetic mice, high-fat feeding resulted in a mild increase in blood glucose levels compared to nondiabetic mice on the standard diet that maintained their blood glucose levels around 120 mg/dl throughout the 8-week study (Figure 1B). As early as 1 week following STZ-injection, diabetic mice had significantly higher blood glucose levels compared to their nondiabetic counterparts (Figure 1B). However, the combination of diabetes and the high-fat diet resulted in significantly lower blood glucose levels compared to diabetic mice on the standard diet, but glucose levels were still higher than both nondiabetic groups (Figure 1B). Insulin levels were significantly lower in both diabetic groups compared to the nondiabetic groups on the equivalent diet (Figure 1C). Finally, high-fat feeding induced hyperinsulinemia in nondiabetic mice (Figure 1C).

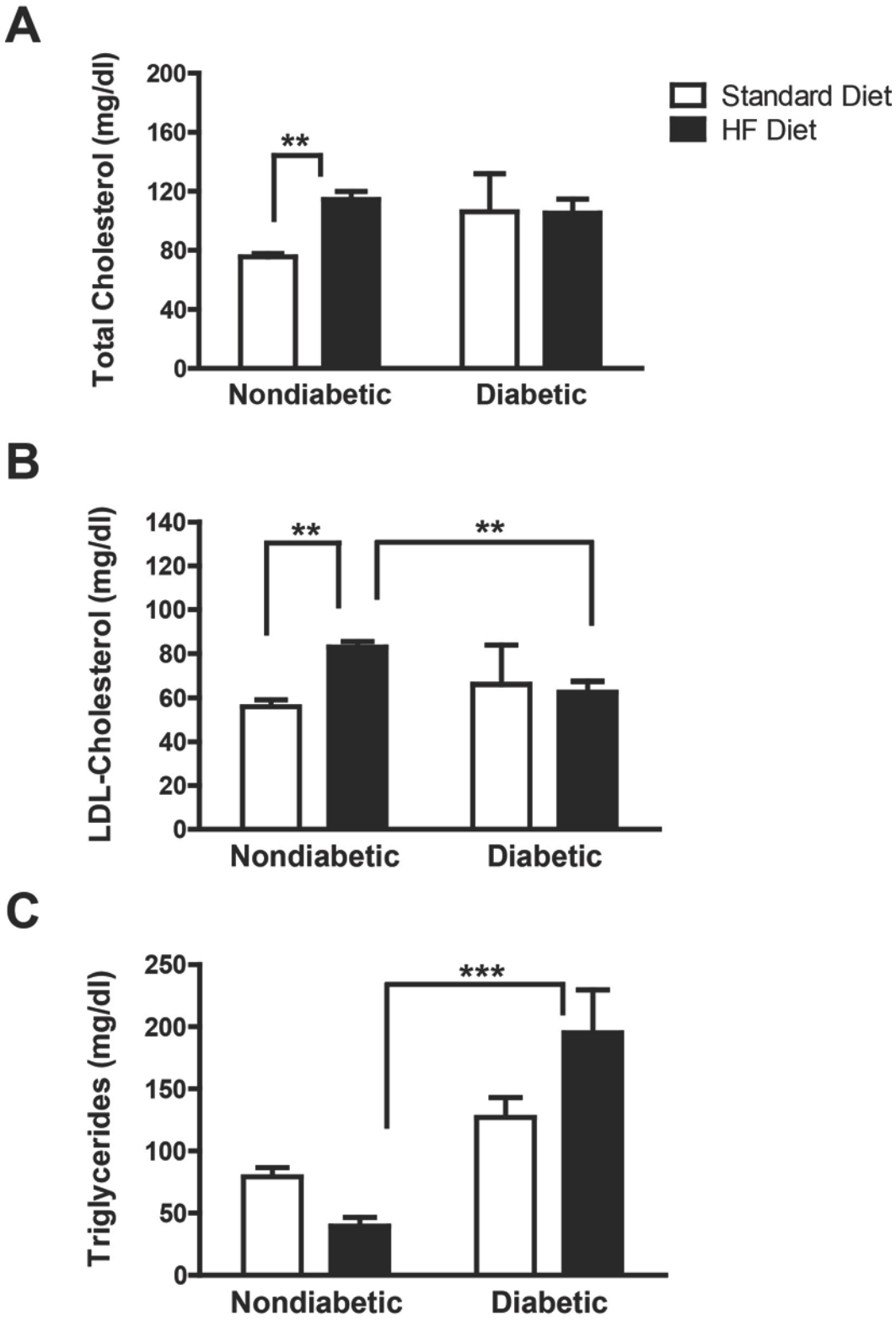
A High-Fat Diet Induces Dyslipidemia

Nondiabetic mice fed a high-fat diet had higher total cholesterol levels compared to nondiabetic mice fed a standard diet (Figure 2A). However, diabetes did not significantly alter total cholesterol in mice fed the standard or high-fat diet compared to their nondiabetic counterparts (Figure 2A). The high-fat diet increased LDL-C in nondiabetic mice, which mirrored the effect of high-fat feeding on total cholesterol in nondiabetic mice (Figure 2A-B). Diabetes did not alter LDL-C levels in mice fed the standard diet compared to their nondiabetic counterparts (Figure 2B). Triglyceride levels were not significantly affected by the high-fat diet in nondiabetic mice (Figure 2C). Diabetic mice on a standard diet had slightly higher triglycerides compared to their nondiabetic counterparts, while triglycerides were significantly

Figure 2: Serum lipids.

Blood was collected at 8 wks post-STZ from non-fasted mice. Total Cholesterol (A), LDL-Cholesterol (B), and Triglycerides (C). Data are presented as means ± SEM (n = 3 – 8 mice per group). $^{**}P < 0.01$ and $^{***}P < 0.001$.

Figure 2


A
Total Cholesterol (mg/dl)
200
160
120
80
40
0
**
Nondiabetic
Diabetic
Standard Diet
HF Diet
B
LDL-Cholesterol (mg/dl)
140
120
100
80
60
40
20
0
**
**
Nondiabetic
Diabetic
C
Triglycerides (mg/dl)
250
200
150
100
50
0
***
Nondiabetic
Diabetic

higher in the diabetic high-fat fed group compared to the nondiabetic high-fat fed group (Figure 2C).

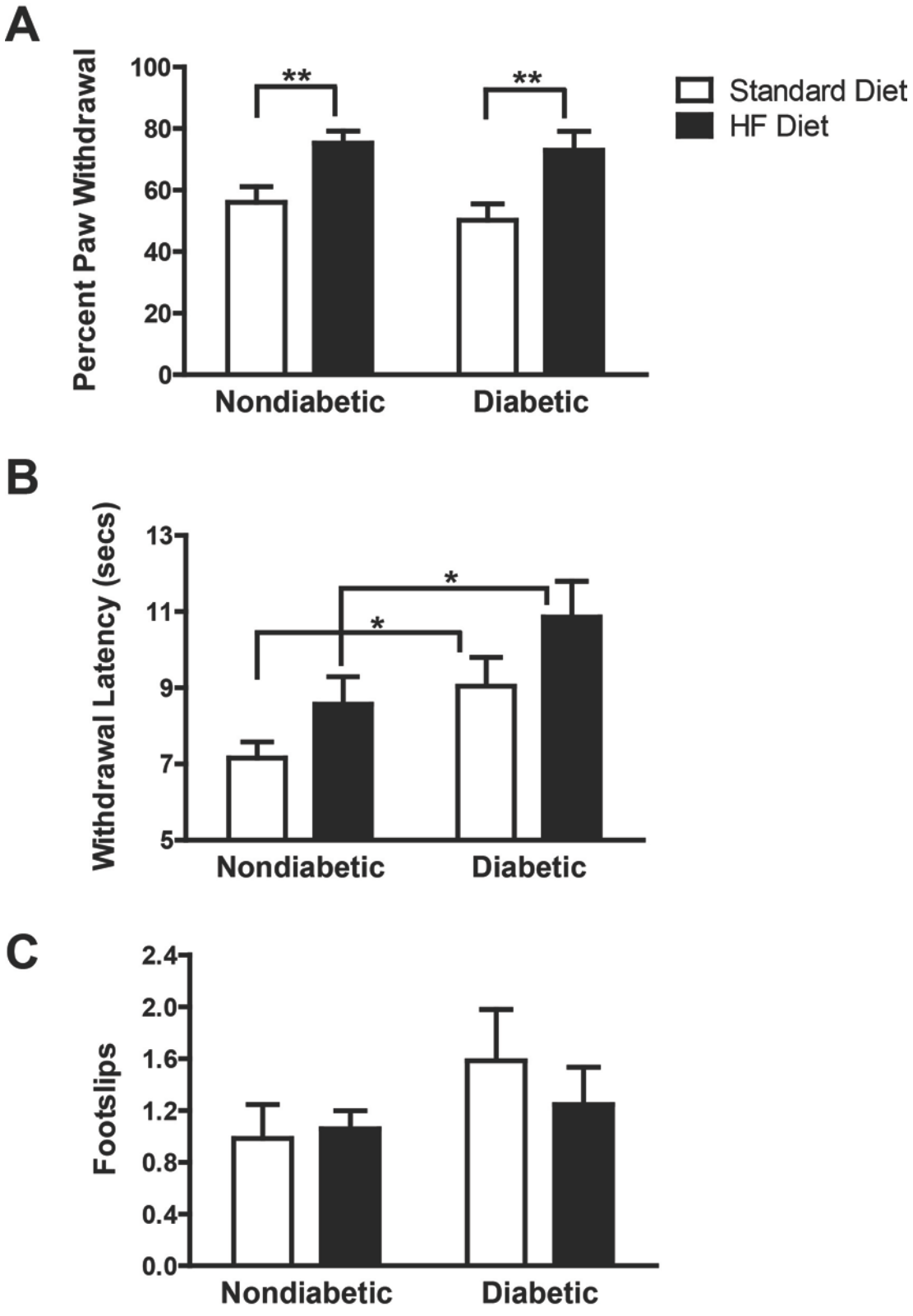
Sensorimotor Behavior is Altered by High-Fat Feeding

Our previous studies of this murine strain have consistently demonstrated that STZ-induced diabetic C57Bl/6 mice develop a slowly progressive insensate neuropathy characterized by a reduction in mechanical sensitivity that appears after 4 weeks of diabetes. However, the degree to which individual cohorts of mice develop a loss of sensation has been variable, ranging from 27-50% change in sensitivity, and we have never observed mechanical hyperalgesia with this model, and mouse strain, and diet. [181-184]. In the current cohort of mice, mechanical sensitivity was reduced by 11% (Figure 3A), however, the pattern of mechanical sensitivity remained similar to previous studies. In striking contrast, the high-fat diet increased mechanical sensitivity in both nondiabetic and diabetic mice compared to their counterparts fed a standard diet by 35% and 45%, respectively (Figure 3A). Diabetes significantly reduced thermal sensitivity in mice on the standard diet but was not significantly worsened by the addition of the high-fat diet (Figure 3B). However, diabetic mice fed the high-fat diet did have significantly lower thermal sensitivity than high-fat fed nondiabetic mice (Figure 3B). A beam walk task was used to assess sensorimotor ability at 8 weeks post-STZ. The number of hind paw slips measured while mice traversed a wooden beam was used to assess sensorimotor deficits and the number of slips was not significantly different among any groups (Figure 3C).

Figure 3: Sensorimotor Behavior.

A) Mechanical sensitivity was assessed at 8 weeks post-STZ using 12 repeated applications of a 1.4 g von Frey monofilament (n = 15 – 21 mice per group). B) Thermal sensitivity. Mice were placed on a Hargreaves apparatus and a 4.0 V radiant heat source was applied to the hind paw footpad. The time elapsed before the animal withdrew the paw was recorded as withdrawal latency (n = 6 – 13 mice per group). C) Sensorimotor ability was assessed by quantifying mean hind paw footslips as mice traversed a narrow wooden beam (n = 6 - 13mice/group). Data presented as means ± SEM. *P < 0.05 and **P< 0.01.

Figure 3


A
Percent Paw Withdrawal
100
80
60
40
20
0
**
**
Standard Diet
HF Diet
Nondiabetic
Diabetic
B
Withdrawal Latency (secs)
13
11
9
7
5
*
*
Nondiabetic
Diabetic
C
Footslips
2.4
2.0
1.6
1.2
0.8
0.4
0.0
Nondiabetic
Diabetic

Nerve Conduction and Epidermal Innervation

Neither diabetes nor high-fat feeding alone altered sensory nerve conduction velocity (Figure 4A). Conversely, the combination of diabetes and high-fat feeding significantly reduced sensory and motor nerve conduction velocities compared to nondiabetic mice fed the high-fat diet (Figure 4A-B). High-fat feeding increased motor nerve conduction velocity (MNCV) in nondiabetic mice but MNCV was not significantly affected by diabetes alone (Figure B). Consistent with previous studies documenting decreased cutaneous innervation associated with diabetic neuropathy [181-183, 185-189], IENFD was significantly reduced in the hind paw footpad skin after 8 weeks of diabetes (Figure 5A-C). However, the combination of the high-fat diet and diabetes did not significantly alter IENFD (Figure 5C).

Oxidative Stress

Immunohistochemistry was used to quantify the abundance of nitrotyrosine, a marker of oxidative stress, in the lumbar DRG. Neither diabetes nor the high-fat diet significantly altered levels of nitrated tyrosine residues in DRG neuronal cell bodies (Figure 6A-B).

5. Discussion

Recent evidence indicating that dyslipidemia may increase a diabetic patient's risk of developing neuropathy (reviewed in [7]), taken in conjunction with high-fat feeding studies in rodents [5, 23, 190], suggests diet may play an important role in modulating diabetic neuropathy progression and phenotype. Here, our data reveals that a high-fat diet fed to STZ-induced

Figure 4: Diabetic mice fed a high-fat diet exhibit deficits in nerve conduction velocity.

Sensory Nerve Conduction Velocity (A) and Motor Nerve Conduction Velocity (B) were measured at 8 wks post-STZ. Data are presented as means ± SEM (n = 3 – 8 mice per group). *P < 0.05 and ***P < 0.001.

Figure 4

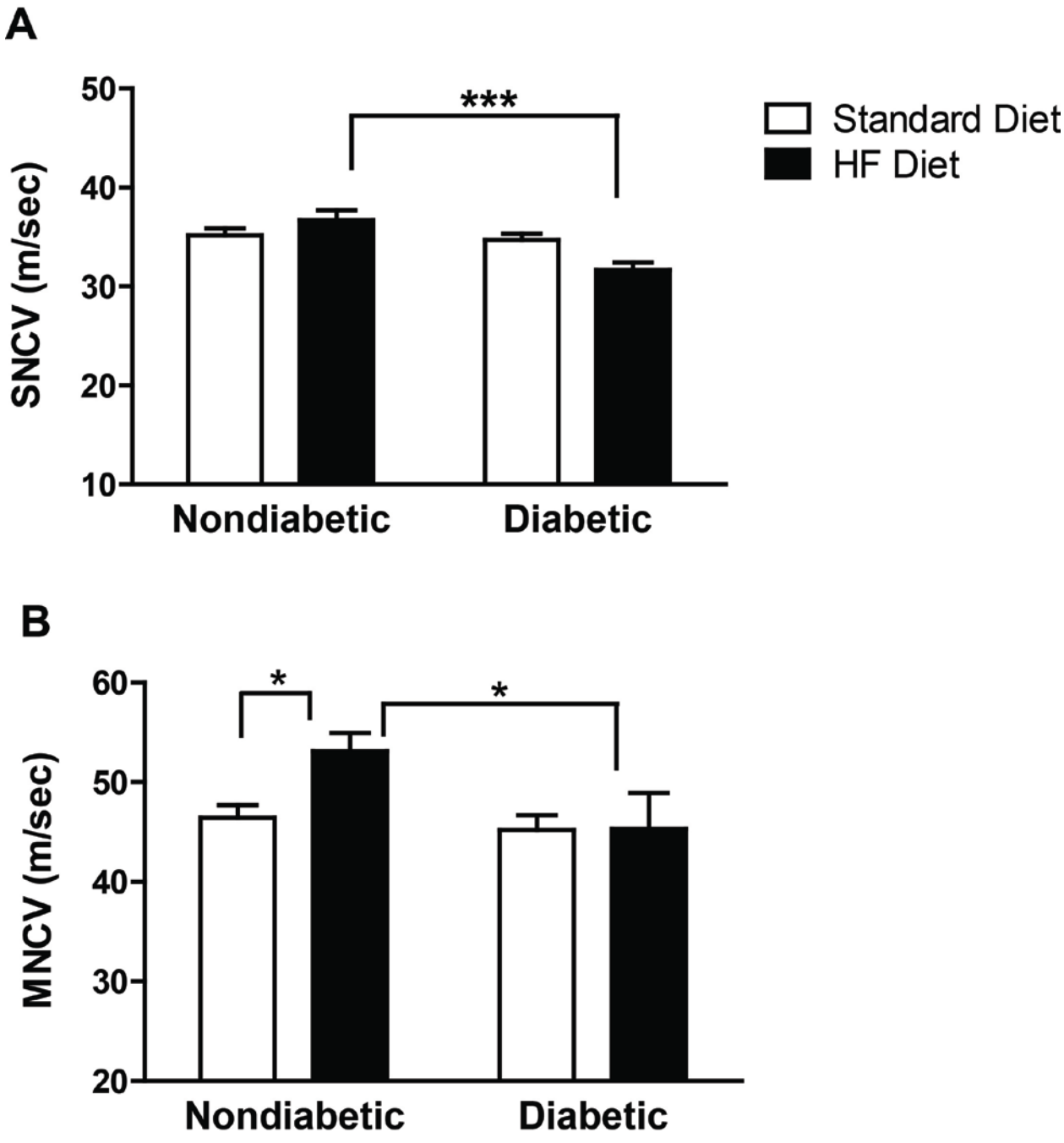

A
50
40
30
20
10
SNCV (m/sec)
***
Standard Diet
HF Diet
Nondiabetic
Diabetic
B
60
50
40
30
20
MNCV (m/sec)
*
*
Nondiabetic
Diabetic

Figure 5: Intraepidermal nerve fiber density is reduced in diabetic mice fed a standard diet.

Plantar skin of the hindpaw was dissected at 8 wks post-STZ. Immunohistochemistry for PGP 9.5 was used to visualize and count nerve fibers that cross the epidermal/dermal border. A-B: Representative images showing a section of plantar skin used to quantify IENFD. A) Nondiabetic Standard Diet. B) Diabetic Standard Diet. Arrows indicate individual axons within the epidermis. Scale bar = 50 μM. C) Quantification of IENFD (n = 11-12 mice per group). Data presented as means ± SEM. *$P < 0.05$

Figure 5

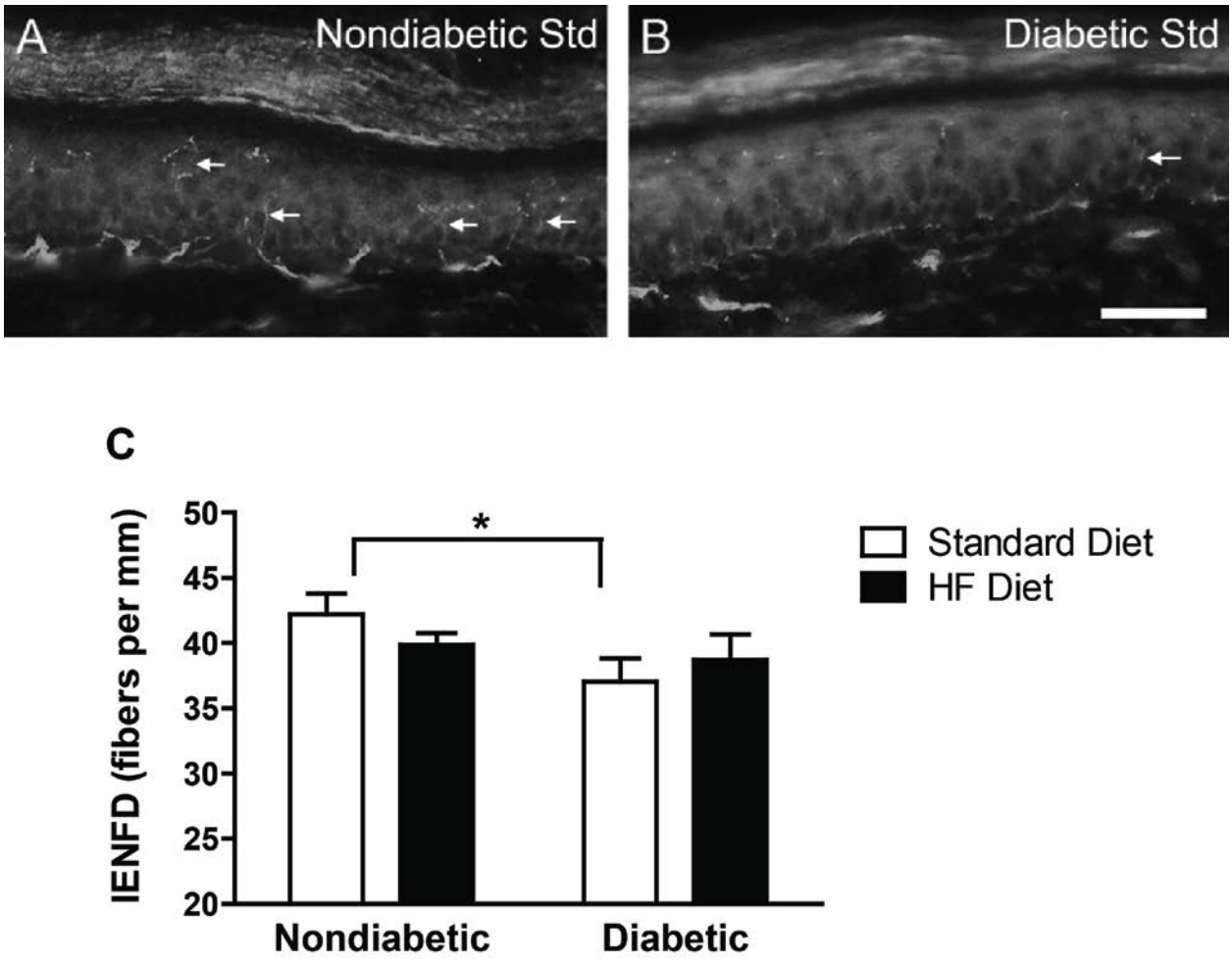

A
Nondiabetic Std
B
Diabetic Std
C
IENFD (fibers per mm)
50
45
40
35
30
25
20
*
Nondiabetic
Diabetic
Standard Diet
HF Diet

Figure 6: Nitrotyrosine expression is increased in lumbar DRG neurons of diabetic mice fed a standard diet.

Quantification of average nitrotyrosine fluorescence intensity per neuron in the lumber DRG. Mean fluorescence intensity was calculated for a total of approximately 10 cells per image from six images per mouse and group means were calculated with respect cell size. Group means for average nitrotyrosine fluorescence per cell are binned by neuron size according to area^2: A) 0 – 200 μm^2, B) 201 – 400 μm^2, C) 401 – 600 μm^2, and D) $\geq$ 601 μm^2. Data are presented as means ± SEM (n = 3 – 8 mice per group). *P < 0.05 and **P< 0.01.

Figure 6

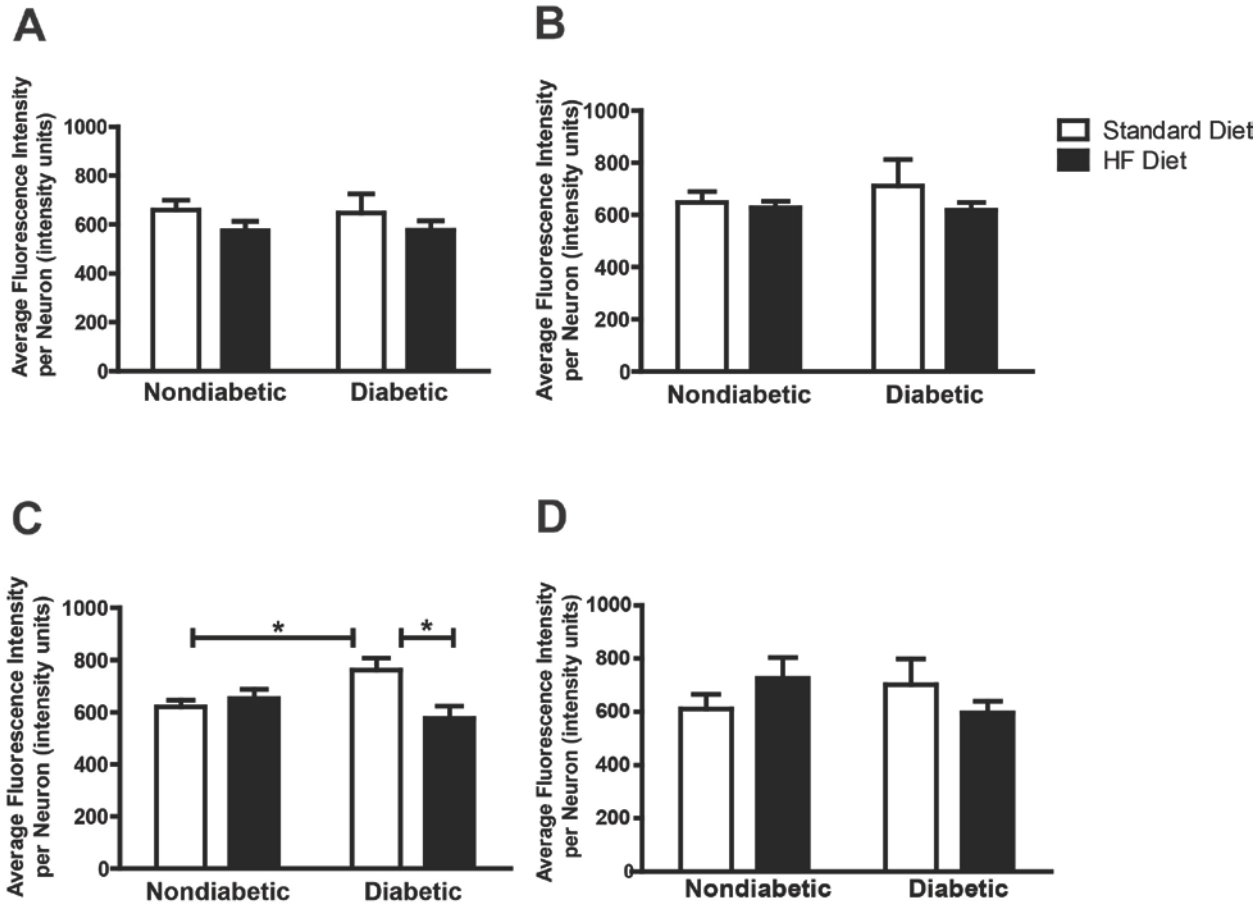
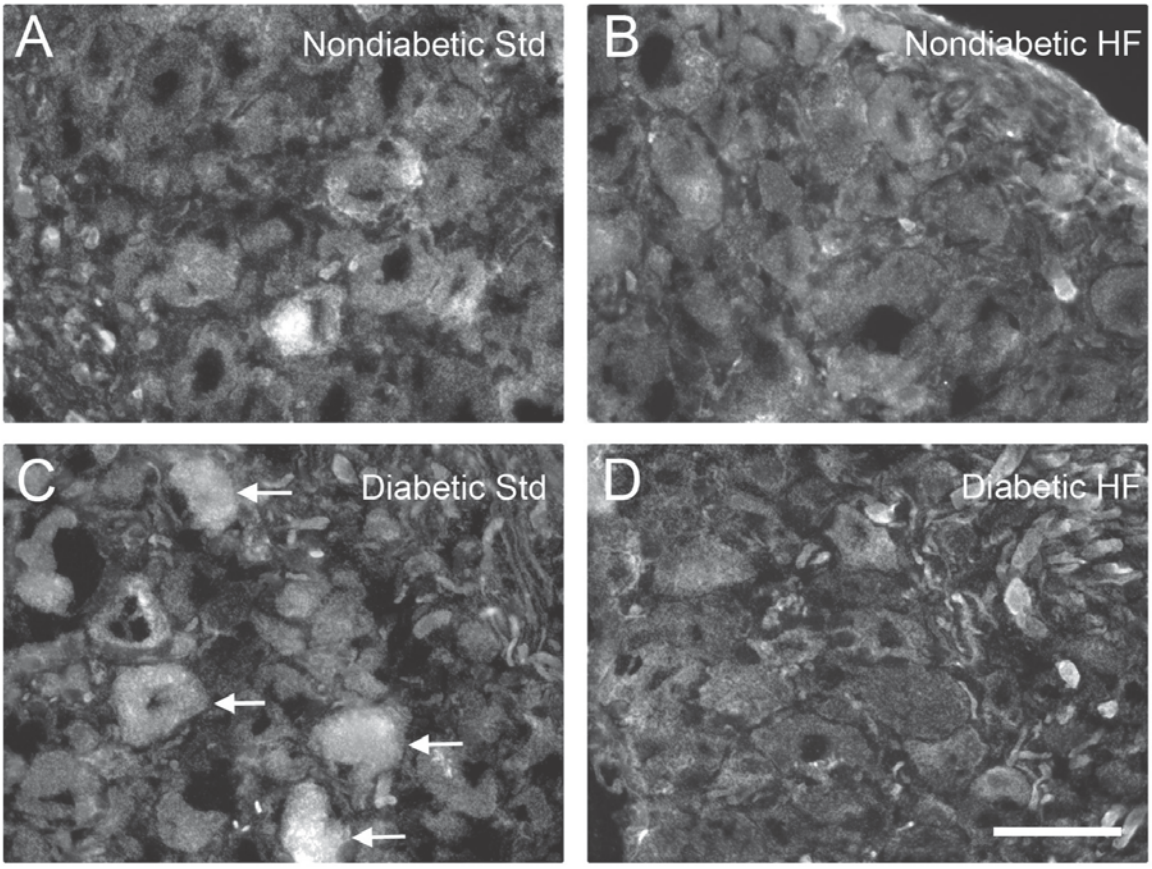

A
B
C
D
Average Fluorescence Intensity per Neuron (intensity units)
1000
800
600
400
200
0
Nondiabetic
Diabetic
Standard Diet
HF Diet
*
*

Figure 7: Representative images showing nitrotyrosine expression in the lumbar DRG.

A) Nondiabetic Standard Diet, B) Nondiabetic High-Fat Diet, C) Diabetic Standard Diet, and D) Diabetic High-Fat Diet. Arrows indicate medium-sized neurons expressing high levels of nitrotyrosine. Scale bar = 50 μM.

Figure 7


A
Nondiabetic Std
B
Nondiabetic HF
C
Diabetic Std
D
Diabetic HF

diabetic C57BL/6 mice significantly alters the phenotype of neural symptoms by inducing a painful neuropathy (mechanical hyperalgesia) instead of an insensate neuropathy (mechanical insensitivity) that normally develops in these diabetic mice [181-184]. In addition, our results support clinical data (reviewed in [7]), that suggests that dyslipidemia may be an independent risk factor for development of diabetic neuropathy.

In numerous studies using this murine strain, STZ dose, and standard diet, our laboratory has reported that STZ-induced diabetic C57Bl/6 mice develop a slowly progressive insensate neuropathy characterized by a reduction in mechanical sensitivity after 4 weeks of diabetes [181-184, 189]. In the current study, we observed a slight reduction in mechanical sensitivity, although the reduction in mechanical sensitivity did not reach statistical significance after 8 weeks of diabetes in this cohort of animals. Importantly, however, STZ-diabetic mice fed a high-fat diet displayed a mechanical hyperalgesia, as opposed to the insensate phenotype typically observed in this inbred mouse strain. The high-fat diet induced a robust mechanical hyperalgesia as mechanical sensitivity was increased by 35% and 45%, respectively, in nondiabetic and diabetic mice compared to their counterparts fed a standard diet. This finding is particularly important because it suggests that diet modulates diabetic neuropathy phenotype in rodents, thus dietary manipulation could be used as a new tool to investigate mechanisms that cause some patients to experience insensate symptoms while others have painful symptoms. In addition, this data is consistent with previous studies that reported that a high-fat diet induces neuropathy in nondiabetic mice [5, 23].

Diet-induced changes in neuropathy were not uniform across modalities or symptoms. For example, diabetes significantly reduced epidermal innervation as previously noted [181-183]; however, the high-fat diet alone or in combination with diabetes did not further affect

IENFD. Similarly, impaired thermal sensitivity was not exacerbated by the high-fat diet in STZ-diabetic mice. This suggests that diabetes alone may be the driving factor underlying reduced IENFD and thermal hypoalgesia in this model.

Neither diabetes nor the high-fat diet significantly altered gait and balance as assessed by the beam walk task. Body weight differences due to the high-fat diet appeared to affect the beam walk task that potentially confounded this data, increased error, and reduced our ability to detect statistically significant differences in hind paw footslips. In addition, gait and balance deficits detected by a beam walk task are thought to be, in part, attributed to altered muscle spindle group Ia innervation [180], which may not be affected after only 8 weeks of diabetes.

Behavioral responses to sensorimotor tests incorporate inputs from multiple axonal fiber types within peripheral nerves. The von Frey method of assessing mechanical sensitivity stimulates a combination of large myelinated A-beta fibers that are sensitive to light touch and small unmyelinated C fibers that are pain-responsive. The Hargreaves test stimulates pain and temperature-sensing C and A-delta fibers, whereas the beam walk test likely assesses contributions from dermal A-fibers and muscle afferent fibers innervating muscle spindles, both of which are important for gait, balance, and proprioception. IENFD is an excellent tool for quantifying small fiber loss, is often used when diagnosing diabetic neuropathy in humans, and has been shown to correlate with diabetes duration in human patients [17, 180, 185, 191]. Slowed SNCV has been thought to reflect conduction deficits predominately in large fibers [180], but reports documenting diabetes-induced slowed SNCV vary as some studies report decreases [192] while others report no change [180, 193]. The diabetic environment affects each fiber population differently and the type of fibers predominantly affected varies with mouse strain and in humans.

In the current study, the high-fat diet induced mechanical hyperalgesia, but diabetes-induced deficits in thermal sensitivity were only mildly worsened by the addition of the high-fat diet. Neither diabetes nor the high-fat diet significantly altered gait and balance as assessed by the beam walk task. Apart from the robust effects of the high-fat diet on mechanical sensitivity, none of the other behavioral data nor anatomical or physiological assessments performed here suggest that the high-fat diet affects sensorimotor behavior or nerve morphology and function differently than diabetes alone. It will be important to investigate how a high-fat diet alters mechanical sensitivity, and whether this selective effect is related to specific peripheral fiber-type or central processing of mechanical sensitivity in the CNS.

Here, we report that a high-fat diet significantly increased body weight in nondiabetic and diabetic mice compared to their counterparts on a standard diet, suggesting that the metabolic derangements that accompany diet-induced obesity may have harmful effects on nerve fiber function, thereby leading to mechanical hyperalgesia.

In addition, total cholesterol and LDL-C were significantly increased in high-fat fed nondiabetic mice compared to their nondiabetic counterparts on the standard diet and triglyceride levels were significantly higher in STZ-diabetic mice fed the high-fat diet compared to their nondiabetic counterparts on the high-fat diet. Because at least one parameter of the lipid profile (serum total cholesterol, LDL-C, or triglycerides) was increased in high-fat fed nondiabetic and STZ-diabetic mice, both groups could be considered dyslipidemic. Thus, diet-induced dyslipidemia may be an underlying factor that drives mechanical hyperalgesia in rodents. Consistent with clinical evidence suggesting dyslipidemia is associated with diabetic neuropathy onset and progression (reviewed in [33]),[34, 35], our data supports the view that diet-induced

dyslipidemia may induce neuropathy in pre-diabetic patients and modulate diabetic neuropathy onset, progression, and/or phenotype.

In the current study, nondiabetic mice fed the high-fat diet exhibited pre-diabetes characterized by elevated glucose levels and hyperinsulinemia. As expected, glucose was significantly higher in both diabetic groups compared to their nondiabetic counterparts. However, glucose levels were lower in high-fat fed diabetics compared to diabetic mice on the standard diet which is most likely explained by the lower percentage of total kilocalories derived from carbohydrate in the high-fat diet (24%) versus the standard diet (40%). STZ is a selective a beta islet cell toxin that kills the insulin producing cells in the pancreas, thus, as expected, insulin levels were significantly lower in both diabetic groups compared to their nondiabetic counterparts. Although high-fat feeding induced mechanical hyperalgesia in both nondiabetic and diabetic mice, nondiabetic mice were hyperinsulinemic whereas diabetic mice remained hypoinsulinemic after 8 weeks of high-fat feeding. Furthermore, diabetic mice on the high-fat diet had significantly higher glucose levels than their nondiabetic counterparts but mechanical sensitivity was not different between these groups. Thus, our data suggests that glucose and insulin levels may not be key factors driving high-fat diet induced mechanical hyperalgesia observed in the current study.

A high-fat diet and diabetes alone have consistently been shown to increase oxidative stress in rodents [5, 72, 190, 194, 195] and increased oxidative stress has been proposed as a mechanism that contributes to pathogenesis of diabetic neuropathy (reviewed in [18, 196, 197]). Vincent and colleagues reported that dyslipidemia leads to high levels of oxidized low-density lipoproteins (ox-LDLs) in mice and *in vitro* and oxLDLs lead to severe DRG neuron oxidative stress and neuron injury, thus identifying a potential mechanism by which dyslipidemia may

contribute to the development of diabetic neuropathy [5]. However, nitrotyrosine levels in the DRG were not altered by diabetes or the high-fat diet in the current study. These results should be interpreted with caution because we only quantified one oxidative stress marker in one tissue (DRG), and this was a short-term study that lasted only 8 weeks. Although nitrotyrosine levels were not affected in the DRG, it is possible that diabetes and/or the high-fat diet increased oxidative stress and these effects may have been more apparent if we had quantified additional oxidative stress markers (i.e. hydroxyoctadecadienoic acid, 4-hydroxynonenal, dityrosine) in the DRG or other tissues such as plasma, isolated LDLs, or sciatic nerve.

Little is known about the mechanisms contributing to high-fat diet-induced painful neuropathy, but one recent study indicates that an extract from the Artemisia plant (known for its anti-inflammatory and anti-nociceptive properties) alleviates high-fat diet induced mechanical hyperalgesia and reduces 12/15 lipoxygenase (regulates pro-inflammatory cytokine production) upregulation, suggesting that inflammation may play a role in high-fat diet induced neuropathy [190]. Pro-inflammatory cytokines and chemokines have been widely implicated in chronic pain and are thought to contribute to the central sensitization that results in mechanical hyperalgesia [198-201]. Importantly, obesity is associated with chronic low-grade inflammation [79, 80], and a high-fat diet increases the pro-inflammatory cytokines IL1-β, tumor necrosis factor alpha (TNF-α), C-reactive protein (CRP), and IL1-6, and stimulates inflammatory signaling in adipose, serum, liver, and brain [124, 202-205]. Along with glia and macrophages [206-211], adipose is an important source of pro-inflammatory cytokines that may upregulate cytokine production due to increased adipose resulting from a high-fat diet [124, 202]. Therefore, a pro-inflammatory environment in the spinal cord may be a key mechanism in the development of mechanical

hyperalgesia associated with diabetes and/or a high-fat diet. Future studies will investigate the role of spinal inflammation in high-fat diet induced mechanical hyperalgesia.

Conclusions

These results demonstrate that a high-fat diet fed to STZ-induced diabetic C57BL/6 mice significantly alters the phenotype of neural symptoms by inducing a painful neuropathy (mechanical hyperalgesia) instead of the insensate neuropathy (mechanical insensitivity). In addition, our results support previous studies [5, 23] that show a high-fat diet fed to nondiabetic mice can induce pre-diabetes and neuropathy. In conclusion, dyslipidemia resulting from a high-fat diet, may modify diabetic neuropathy phenotype and/or increase risk for developing diabetic neuropathy. Furthermore, the metabolic defects that accompany diet-induced obesity may have harmful effects on nerve fiber function thereby leading to mechanical hyperalgesia. These results may provide some insight into why some patients develop painful versus insensate neuropathy.

Although the ability of a high-fat diet to induce mechanical hyperalgesia is novel, little is known about the mechanisms responsible for this robust painful diabetic neuropathy phenotype in this model, thus further study is warranted. Positive outcomes from future studies could lead to dietary modifications and/or increased use of treatments that improve dyslipidemia (i.e. omega 3 fatty acid supplementation, statins, or exercise) as therapeutic interventions for patients with painful diabetic neuropathy.

CHAPTER 3

Mitochondrial Function In Diabetic Neuropathy

1. Abstract

Mitochondrial dysfunction has been proposed as a contributory mechanism driving diabetic neuropathy. In contrast, several recent studies suggest that mitochondrial dysfunction is subtle and occurs in late stages of diabetes. Experiments here used a high-fat diet to exacerbate the severity of diabetes and to expedite the time-course in which mitochondrial dysfunction occurs. We hypothesized that a high-fat diet in addition to diabetes would create added stress to sensory neurons and exacerbate mitochondrial dysfunction. Mitochondrial respiration measurements and expression of proteins involved in oxidative phosphorylation or mitochondrial function were quantified in the dorsal root ganglia. Comparisons were made between nondiabetic and STZ-induced diabetic C57Bl/6 mice consuming a standard or high-fat diet for 8 weeks. Basal mitochondrial respiration was significantly reduced in diabetic mice fed a standard diet compared to their nondiabetic counterparts. There were no differences among groups in leak dependent respiration, maximal respiration, spare respiratory capacity, or expression of subunits from oxidative phosphorylation complexes I, II, or V. In contrast, Complex III subunit Core-2 and voltage dependent anion channel (VDAC) were significantly increased in diabetic mice compared to nondiabetic mice fed the standard diet ($p < 0.05$). There were also no differences among groups in UCP2, PGC-1α, PGC-1β levels or Akt, mTor, or AmpK activation. These data suggest subtle mitochondrial dysfunction and potential compensatory mitochondrial biogenesis occurs after 8 weeks of STZ-induced diabetes and these parameters are not altered by high-fat feeding. Our results support the idea that mild mitochondrial dysfunction occurs early in the diabetic progression suggesting that mitochondrial impairment may play a role in the dying-back type axonal degeneration that occurs in diabetic neuropathy.

2. Introduction

Neurons, due to their relatively high metabolic demands, rely heavily on mitochondrial energy production, which is required for many cellular processes including protein synthesis, growth and survival, and neuronal excitability [59]. Consequently, metabolic disturbances that alter normal mitochondrial function are especially detrimental to neurons [59]. Thus, it is not surprising that mitochondrial dysfunction has been implicated in the pathogenesis of many neurological disorders [61].

Mitochondrial dysfunction has been identified an important etiological factor in the pathogenesis of diabetic neuropathy, but the contributions of mitochondrial dysfunction in diabetic neuropathy remain controversial [20, 22, 63, 64, 66, 73]. Diabetes has been shown to attenuate mitochondrial respiration and spare respiratory capacity, reduce activities of mitochondrial enzyme complexes I and IV, and decrease expression of mitochondrial oxidative phosphorylation proteins in cultured DRG isolated from diabetic animals compared to nondiabetics [63, 64, 66]. In addition, cultured DRG neurons from diabetic animals exhibit mitochondrial accumulation in axon swellings and altered mitochondrial fission-fusion equilibrium resulting in small mitochondria with ultrastructural abnormalities [67-70]. Furthermore, a distal dying-back axonal degeneration is predominant in Charcot-Marie-Tooth disease type 2 (CMT2) and mutations in the mitochondrial fusion protein, mitofusin 2, are the most common cause of CMT2 that has been identified to date [212]. Together, these results suggest that mitochondrial damage and dysfunction may play a role in the distal dying-back neurodegeneration that occurs in diabetic neuropathy. However, although diabetes has been shown to alter mitochondrial function, evidence that mitochondrial dysfunction occurs in diabetic models that exhibit significant characteristics of neuropathy is lacking [20, 22, 66, 73].

Studies discussed above demonstrated diabetes-induced mitochondrial dysfunction, but in all but one study [64], observations were made in models of diabetic neuropathy that lacked significant sensory-related behavioral changes and did not show reduced epidermal innervation, or neuropathy was not characterized [20, 22, 66, 68, 73]. Thus, further investigation using models of diabetic neuropathy that exhibit significant indices of neuropathy are needed to further elucidate the role of diabetes-induced mitochondrial dysfunction in diabetic neuropathy.

Because the prevalence of overweight, obesity, and physical inactivity continue to increase in our society, the influence of lifestyle-related metabolic variables (i.e. diet and the lipid profile) have become increasingly important in terms of diabetic neuropathy risk in human patients. According to the National Health and Nutrition Examination Survey (NHANES) 1999-2002, 85.2% of individuals with diagnosed diabetes were overweight or obese [213]. As the prevalence of diabetes has risen significantly over the last decade, it is likely the prevalence of overweight and obesity in diabetic patients has also increased [1]. Although obesity is a concomitant condition in the majority of diabetic patients, rodent studies of diabetic neuropathy have typically been conducted in lean animals fed a standard, low-fat diet (≤ 14% kcals from fat).

To more closely emulate human condition, we used a high-fat diet in conjunction with diabetes to examine the effect of diet on neuropathy progression and phenotype. Our previous data reveals that a high-fat diet fed to STZ-induced diabetic C57BL/6 mice altered the phenotype of neural symptoms by inducing a mechanical hyperalgesia instead of the mechanical insensitivity that normally develops in this model of diabetic neuropathy [30]. Interestingly, high-fat fed nondiabetic mice also developed mechanical hyperalgesia. Previous rodent studies suggest changes in mitochondrial respiration and gene expression are relatively slow, subtle, and

do not occur until later stages of diabetes, suggesting hyperglycemia alone may not be driving mitochondrial changes in the DRG [64]. Thus, it is important to characterize mitochondrial function in this clinically relevant model with a robust behavioral phenotype. Here, we examine mitochondrial dysfunction in the DRG as a potential mechanism underlying high-fat diet induced neuropathy in nondiabetic and STZ-induced type 1 diabetic mice.

3. Experimental Procedures

Animals and Diet

In this study, animal age, housing conditions, and diets were the same as described in Chapter 2. Seven week-old male C57Bl/6 mice were purchased from Charles River (Wilmington, MA), housed two mice per cage under pathogen free conditions, and placed on a 12:12h light/dark cycle.. All animals had ad libitum access to food and water and were fed a standard diet or high-fat diet. Animals in the high-fat diet group were fed the standard diet for 1 week prior to STZ-injection and began the high-fat diet 3 hours post STZ-injection. All protocols and procedures were approved by the University of Kansas Medical Center Animal Use and Care Committee.

Diabetes Induction and Glucose Measurement

The diabetes induction, body weight, and glucose measurement protocols used in these experiments were identical to the protocol described in Ch. 2. A single intraperitoneal STZ injection (180 mg/kg body weight) in sodium citrate buffer was administered to 8 week-old C57Bl/6 mice to induce diabetes. Eight week-old nondiabetic C57Bl/6 mice were injected with

vehicle (sodium citrate) buffer. All mice were fasted for 3 hours pre and post STZ-injection. Body weight and blood glucose were monitored beginning 1 week post-STZ injection and every week thereafter. Mice with blood glucose levels of > 300 mg/dl (> 16 mmol/L) were considered diabetic. STZ-injected mice with blood glucose levels below 300 mg/dl were excluded from the study. Treatment groups are abbreviated throughout as follows: nondiabetic standard diet (NdStd); nondiabetic high-fat diet (NdHF); diabetic standard diet (DbStd); and diabetic high-fat diet (DbHF).

Mitochondrial Respiration Measurement

Mitochondrial respiration was calculated by systematic inhibition and activation of specific components of the electron transport chain (ETC) and oxidative phosphorylation machinery using a dual-chambered respirometer (Oro Boros Oxygraph 2K, Innsbruck, Austria). At 8 weeks post-STZ or vehicle injection, mice were anesthetized with isofluorane, decapitated, and l2 lumbar DRG and peripheral axonal processes were dissected. Immediately following dissection, tissue from each individual mouse was incubated separately at 37^0 C in 2 ml of Dulbecco's modified eagle culture medium (DMEM # 11966, Gibco-Invitrogen, Carlsbad, CA), containing 2.5 mM glucose, 1 mM pyruvate, and 1mM GlutaMAX (Gibco) in the O_2 chamber. Basal respiration rate was measured after VO_2 flux (O_2 consumption rate, pmols•sec^{-1}•ml^{-1}) stabilized following tissue addition. Next, ATP synthase was inhibited by injection of 3 µl of oligomycin (100 mM, Sigma Aldrich) into the O_2 chamber via Hamilton syringe and the resultant VO_2 flux was used to calculate leak dependent mitochondrial respiration. After O_2 flux stabilized following oligomycin injection, 2 µl FCCP (3 mM, trifluorobarbonylcyanide phenylhydrazone, Sigma Aldrich), was injected into the O_2 chamber. FCCP permeabilizes the

inner mitochondrial membrane to protons, ameliorates the proton gradient and subsequently uncouples electron transport and oxidative phosphorylation, thus allowing the maximal mitochondrial respiration rate to be calculated. Finally, 4 µl of antimycin (5 mM, Sigma Aldrich) was injected to inhibit complex III of the ETC, thus inhibiting mitochondrial respiration. The resultant rate equals non-mitochondrial respiration. Basal respiration, leak dependent respiration, and spare respiratory capacity were calculated after subtracting the non-mitochondrial respiration rate (O_2 flux). Spare respiratory capacity was calculated by subtracting the basal O_2 flux rate from the maximal O_2 flux rate. Immediately following respiration experiments, tissue and culture media were removed from the chamber, centrifuged briefly to pellet, media was aspirated, and tissue was snap frozen in liquid nitrogen and stored at -80^0 C until prepared for protein extraction.

Tissues were lysed by sonication in Cell Extraction Buffer (Invitrogen) containing 55.55 µl/ml protease inhibitor cocktail, 200mM Na_3VO_4, and 200mM NaF. Following sonication, protein was extracted while tissue lysates were kept on ice for 1 hour and vortexed every 10 minutes. Samples were then centrifuged at 10,000 *g* for 10 minutes at 4^0 C and the protein concentration of the supernatant was measured using the Bio-Rad protein assay based on the Bradford method (Bio-Rad, Hercules, CA). Total protein concentration and VDAC band intensity (VDAC/actin arbitrary units, from Western blot) were used to normalize mitochondrial respiration data (pmols $O_2 * sec^{-1} * mg\ protein^{-1} * VDAC^{-1}$).

Western Blots

Samples were boiled at 95-100 ^{0}C with Lane Marker Reducing Sample Buffer (Thermo Scientific, Waltham, MA) for 3 minutes. Samples containing 25µg of protein were separated by electrophoresis (35 mA/gel, 0.75 hr, 4^0 C) on 4-15% gradient tris-glycine polyacrylamide gels (Bio-Rad) and transferred onto nitrocellulose membrane (400 mA, 1.5 hr, 4^0C). Nitrocellulose membranes were blocked for 1 hour at room temperature in blocking solution (5% non-fat powdered milk and 0.05% Tween-20 in 0.1 M phosphate buffered saline [PBS, pH 7.4]) followed by overnight incubation (4^0C) in primary antibody diluted in blocking solution (1% non-fat powdered milk and 0.05% 0.1 M PBS [pH 7.4])**.** The following primary antibodies were used to probe membranes: Total Ox Phos Antibody Cocktail MS601 (1:200, Mito Sciences, Eugene Oregon), total Akt (1:1,000, Cell Signaling), phospho (Ser473) Akt (1:500, Cell Signaling), total mTor (1:500, Cell Signaling), phospho-mTor (1:500, Cell Signaling), total AmpK (1: 1,000, Cell Signaling), phospho-AmpK (1:1,000, Cell Signaling), PGC-1α (1:500, Calbiochem), PGC-1β (1:1000, Abcam) Sirt1 (1:400, Abcam), VDAC (1:1,000, Calbiochem), UCP2 (1:500, Abcam) and actin (1:10,000, Millipore).

After incubation in primary antibody, nitrocellulose membranes were then washed in PBST (0.05% Tween-20 in 0.1 M phosphate buffered saline [pH 7.4]) and incubated for 1 hour at room temperature in anti-mouse or anti-rabbit IgG-HRP secondary antibody (Santa Cruz, Santa Cruz, CA) diluted 1:5,000 in blocking solution. Bands were visualized by enhanced chemiluminescence (ECL) using Supersignal West (Femto or Pico) Substrate (Pierce, Rockford, IL) and detected on X-ray film. Bands were quantified via densitometry using NIH Image J software.

In order to normalize band intensity to total protein or determine phospho/total ratio for a specific protein, nitrocellulose membranes were stripped using Restore Plus Western Blot Stripping Buffer (Pierce, Rockford, IL), and probed with actin primary antibody (Millipore, Billerica, MA) or the total primary antibody corresponding to each phospho-antibody. Bands were visualized and quantified as described above.

β-hydroxybutyrate Levels

Serum β-hydroxybutyrate levels were quantified to assess ketosis levels. Blood was collected into Eppendorf tubes, placed on ice to clot for 30 minutes, and centrifuged for 15 minutes at 3,000 x *g*. Serum was removed and stored at -80^{o}C until assayed with β-hydroxybutyrate kit (Pointe Scientific) according to manufacturer's directions.

β-Hydroxybutyrate Treated Cells

SH-SY5Y neuronal cells were cultured in DMEM high glucose (Gibco #11965) supplemented with 10% FBS and 1% penicillin/streptomycin. For experimental procedures, cells were plated in 60mm tissue culture dishes and grown to ~90% confluency, then rinsed with warm PBS, and treated with DMEM in 5mM glucose supplemented with 10% FBS and 1% penicillin/streptomycin +/- 5mM β-hydroxybutyrate (Sigma-Aldrich). After 8 hour in treatment medium, cells were rinsed with ice-cold PBS and cell lysates generated using mammalian protein extraction reagent (Thermo Pierce #78503) per manufacturer's instructions. After quantification of total protein using DC assay (Bio-Rad #500-0112), samples were denatured in Lane Marker Reducing Sample Buffer (Thermo Scientific, Waltham, MA) and subjected to SDS-PAGE

electrophoresis and transferred to nitrocellulose membrane as described above. Twenty µg of protein was loaded per sample lane. Nitrocellulose membranes were probed separately with Total Ox Phos, VDAC, and actin primary antibodies overnight at 4^0C according to Western blot procedures as described above.

Statistics

Data were analyzed using a two-factor analysis of variance (ANOVA) with Fisher's test of least square difference post-hoc comparisons. Pearson correlations using two-tailed P-values were performed to assess the relationship between β-HB levels and paw percent withdrawal and withdrawal latency. Statistical significance was set at $P < 0.05$.

4. Results and Figures

Body Weight, Glucose, and Insulin

These metabolic data were reported in Chapter 2 and are briefly summarized here. Typical with this model of type 1 rodent model of diabetes, diabetic mice weighed less than their nondiabetic counterparts. High-fat fed diabetic mice gained weight compared to diabetics fed the standard diet who lost weight over the course of 8 weeks. Nondiabetic mice fed the high-fat diet were, on average, 10 g heavier than nondiabetic mice fed the standard diet at week 8 of the study. Diabetic mice fed the standard diet consumed at least 2 times as many kcals/day compared to all other groups. Interestingly, daily energy intake was not increased in nondiabetic or diabetic high-fat fed mice, despite increased weight gain when compared to their counterparts fed the standard diet.

Both diabetic groups exhibited severe hyperglycemia, but diabetic mice fed the high-fat diet had significantly lower blood glucose levels than diabetic mice fed the standard diet at throughout the duration of the study. Although hyperglycemia was not severe enough to be defined as overt diabetes, glucose levels were elevated in NdHF compared to NDStd, suggesting nondiabetic mice fed the high-fat diet may be in a pre-diabetic state. As expected in STZ-induced diabetic mice, insulin levels were significantly lower in both diabetic groups compared to the nondiabetic groups on the equivalent diet. High-fat feeding induced hyperinsulinemia in nondiabetic mice, providing additional evidence to suggest that high-fat fed nondiabetic mice have disrupted insulin and glucose homeostasis, consistent with pre-diabetes.

Sensorimotor Behavior is Altered by High-Fat Feeding

As reported in Chapter 1, our previous data reveals that a high-fat diet fed to STZ-induced diabetic C57BL/6 mice strongly alters phenotype of neural symptoms by inducing a robust mechanical hyperalgesia instead of the mechanical insensitivity that normally develops in this model. We have not confirmed specific mechanisms that drive high-fat diet-induced mechanical hyperalgesia, thus it is important to examine the potential role of mitochondrial dysfunction this novel, diet-induced behavioral phenotype.

Mitochondrial Respiration

To determine the effect of diabetes and a high-fat diet on mitochondrial function in mice that exhibit signs of neuropathy, mitochondrial respiration was assessed in the lumbar DRG and peripheral axonal processes. Basal respiration was significantly lower in diabetic mice fed the

standard diet compared to diabetic mice fed the high-fat diet (Figure 1A). There were no significant differences among groups in leak dependent respiration (Figure 1B) or maximal respiration (Figure 1C). Spare respiratory capacity was higher in diabetic mice fed the standard diet compared to nondiabetics consuming the equivalent diet (Figure 1D). The high-fat diet did not alter mitochondrial respiration in nondiabetic or diabetic mice (Figure 1A-D). Despite deficits in basal respiration, no other respiration parameters were diminished in diabetic mice fed the standard diet, suggesting diabetes had a relatively mild effect on mitochondrial respiration.

Expression of Proteins Involved in Mitochondrial Function

Western blots were performed on DRG and peripheral axon lysates to determine if diabetes or high-fat feeding had an effect on the expression of oxidative phosphorylation proteins or other proteins that affect mitochondrial function or biogenesis. Expression of subunits from oxidative phosphorylation complexes I, II, or V were not altered by diabetes or the high-fat diet (Figure 2A-B, D). Uncoupling protein 2 (UCP2) expression was also not different among groups (Figure 3A). In contrast, Complex III subunit Core-2 (Figure 2C) and voltage dependent anion channel (Figure 3B, VDAC) were significantly increased in diabetic mice compared to nondiabetic mice fed the standard diet. A significant increase in both of these proteins in DbStd vs. NdStd suggests diabetes may stimulate mitochondrial biogenesis in face of impaired basal mitochondrial respiration.

However, there were no significant differences among groups in expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) or

Figure 1: Mitochondrial respiration measurements in freshly isolated lumbar dorsal root ganglia.

Twelve lumbar DRG and their peripheral processes were freshly isolated at 8 weeks post-STZ and high-fat diet. Whole intact DRG with processes were placed into the respiration chamber. A) Basal respiration was measured before addition of drugs or inhibitors. B) Leak dependent respiration was measured after oligomycin injection. C) Maximal respiration in response to FCCP injection. D) Spare respiratory was calculated by subtracting basal O_2 flux from maximal O_2 flux. O_2 flux was normalized to total protein and VDAC band intensity from Western blot (VDAC/actin). Data are presented as means ± SEM (n = 8-10 mice per group) . *P < 0.05.

Figure 1

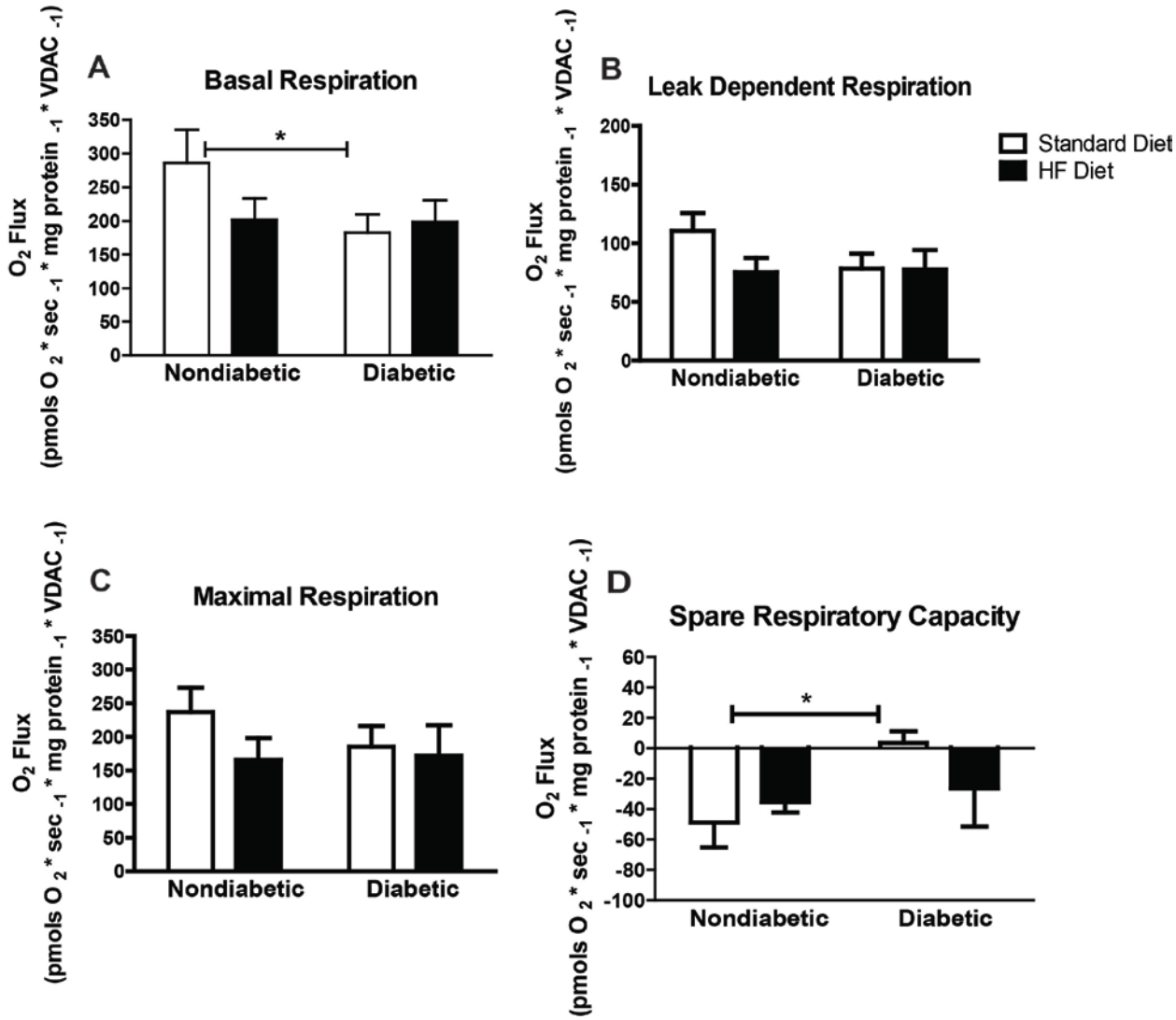

A
Basal Respiration
B
Leak Dependent Respiration
C
Maximal Respiration
D
Spare Respiratory Capacity
O2 Flux
(pmols O 2 * sec -1 * mg protein -1 * VDAC -1)
Nondiabetic
Diabetic
Standard Diet
HF Diet
*

Figure 2: Expression of mitochondrial oxidative phosphorylation proteins in the lumbar dorsal root ganglia.

Mitochondrial oxidative phosphorylation proteins were measured by Western blot. Tissues were harvested at 8 weeks post-STZ and high-fat diet. Representative images and quantification of subunits of mitochondrial oxidative phosphorylation complexes. A) Complex I, B) Complex II, C) Complex III, and D) Complex V. Band intensities were normalized to actin. Data are presented as means ± SEM (n = 8-10 mice per group) . *P < 0.05.

Figure 2

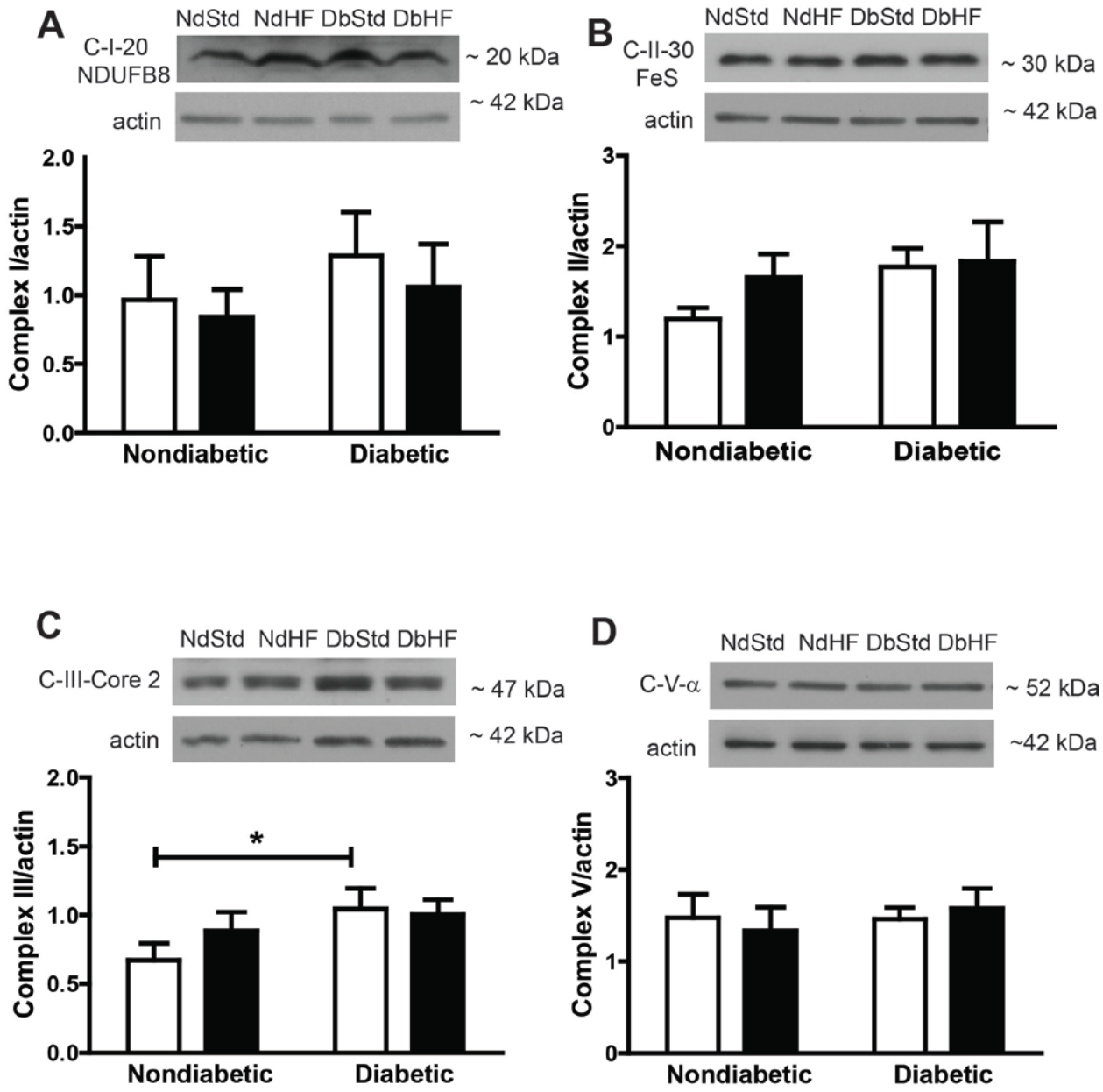
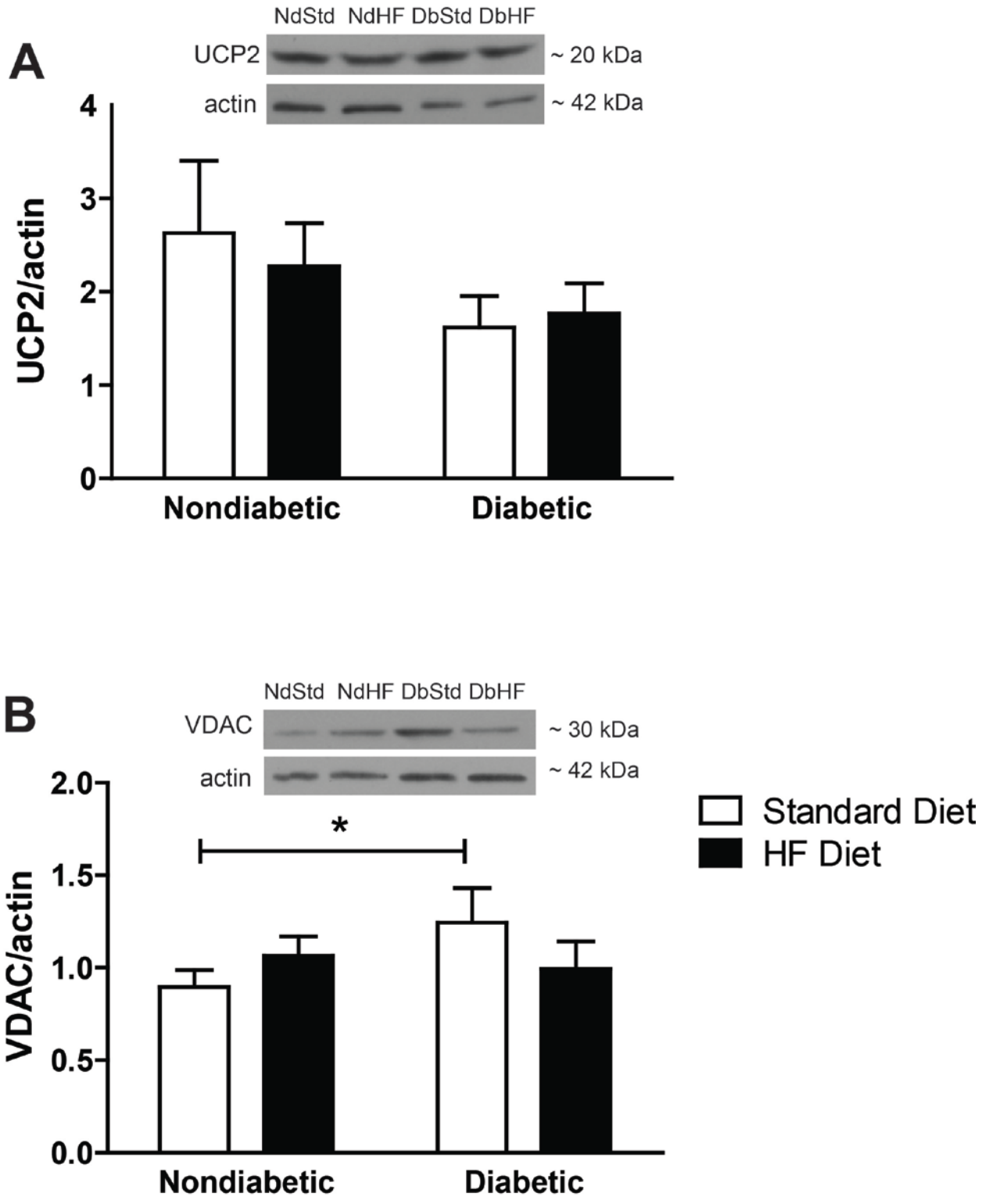

A
NdStd NdHF DbStd DbHF
C-I-20
NDUFB8
~ 20 kDa
actin
~ 42 kDa
Complex I/actin
2.0
1.5
1.0
0.5
0.0
Nondiabetic
Diabetic
B
NdStd NdHF DbStd DbHF
C-II-30
FeS
~ 30 kDa
actin
~ 42 kDa
Complex II/actin
3
2
1
0
Nondiabetic
Diabetic
C
NdStd NdHF DbStd DbHF
C-III-Core 2
~ 47 kDa
actin
~ 42 kDa
Complex III/actin
2.0
1.5
1.0
0.5
0.0
*
Nondiabetic
Diabetic
D
NdStd NdHF DbStd DbHF
C-V-α
~ 52 kDa
actin
~42 kDa
Complex V/actin
3
2
1
0
Nondiabetic
Diabetic

Figure 3: VDAC and UCP2 expression in the lumbar dorsal root ganglia.

Proteins were measured by Western blot. Tissues were harvested at 8 weeks post-STZ and high-fat diet. Representative images and quantification of group means for UCP2 (A) and VDAC (B). Band intensities were normalized to actin. Data are presented as means ± SEM (*n* = 8-10 mice per group). No significant differences among groups.

Figure 3


A
NdStd NdHF DbStd DbHF
UCP2
~ 20 kDa
actin
~ 42 kDa
UCP2/actin
4
3
2
1
0
Nondiabetic
Diabetic
B
NdStd NdHF DbStd DbHF
VDAC
~ 30 kDa
actin
~ 42 kDa
*
VDAC/actin
2.0
1.5
1.0
0.5
0.0
Nondiabetic
Diabetic
Standard Diet
HF Diet

peroxisome proliferator- activated receptor gamma coactivator 1-beta (PGC-1β) which are transcriptional coactivators that regulate mitochondrial biogenesis and function (Figure 4A-B).

Furthermore, there was no significant effect of diabetes or high-fat feeding on activation of proteins indirectly involved in mitochondrial biogenesis via PGC-1α activation or inhibition, including NAD-dependent deacetylase sirtuin-1 (Sirt 1, Figure 4C), activated protein kinase B (phospho Akt, Figure 5A), or 5' adenosine monophosphate-activated protein kinase (AmpK, Figure 5C) Mammalian target of rapamycin (mTor) activation is inhibited during nutrient starvation and its inhibition leads to autophagy. In addition, activated mTor plays an important role in the PGC-1α complex that is essential for activating mitochondrial DNA synthesis. Despite significant weight loss in both diabetic groups, mTor activation was not altered in diabetic mice fed the standard or high-fat diet (Figure 5B).

Diabetic Animals Are in Ketoacidois

Diabetic and high-fat fed mice can develop ketosis due to increased fatty acid metabolism. Ketone bodies provide an alternative energy source and can affect mitochondrial function. Therefore, serum beta-hydroxybutyrate (β-HB) levels were quantified. Circulating β-HB levels were significantly increased in both diabetic groups compared to their nondiabetic counterparts. DbHF mice did not have significantly higher β-HB levels than DbStd mice, but the lack of statistical significance was likely due to high variability in the DbHF group (Figure 6). β-HB levels were not higher in high-fat fed nondiabetic mice compared to nondiabetic mice on the standard diet (Figure 6). The β-HB levels in both groups of diabetic mice were high enough to

Figure 4: Expression of proteins involved in mitochondrial biogenesis in the lumbar dorsal root ganglia.

Proteins were measured by Western blot. Tissues were harvested at 8 weeks post-STZ and high-fat diet. Representative images and quantification of group means for PGC-1α (A), PGC-1β, and Sirt 1 (C). Band intensities were normalized to actin. Data are presented as means ± SEM (*n* = 8-10 mice per group). No significant differences among groups.

Figure 4

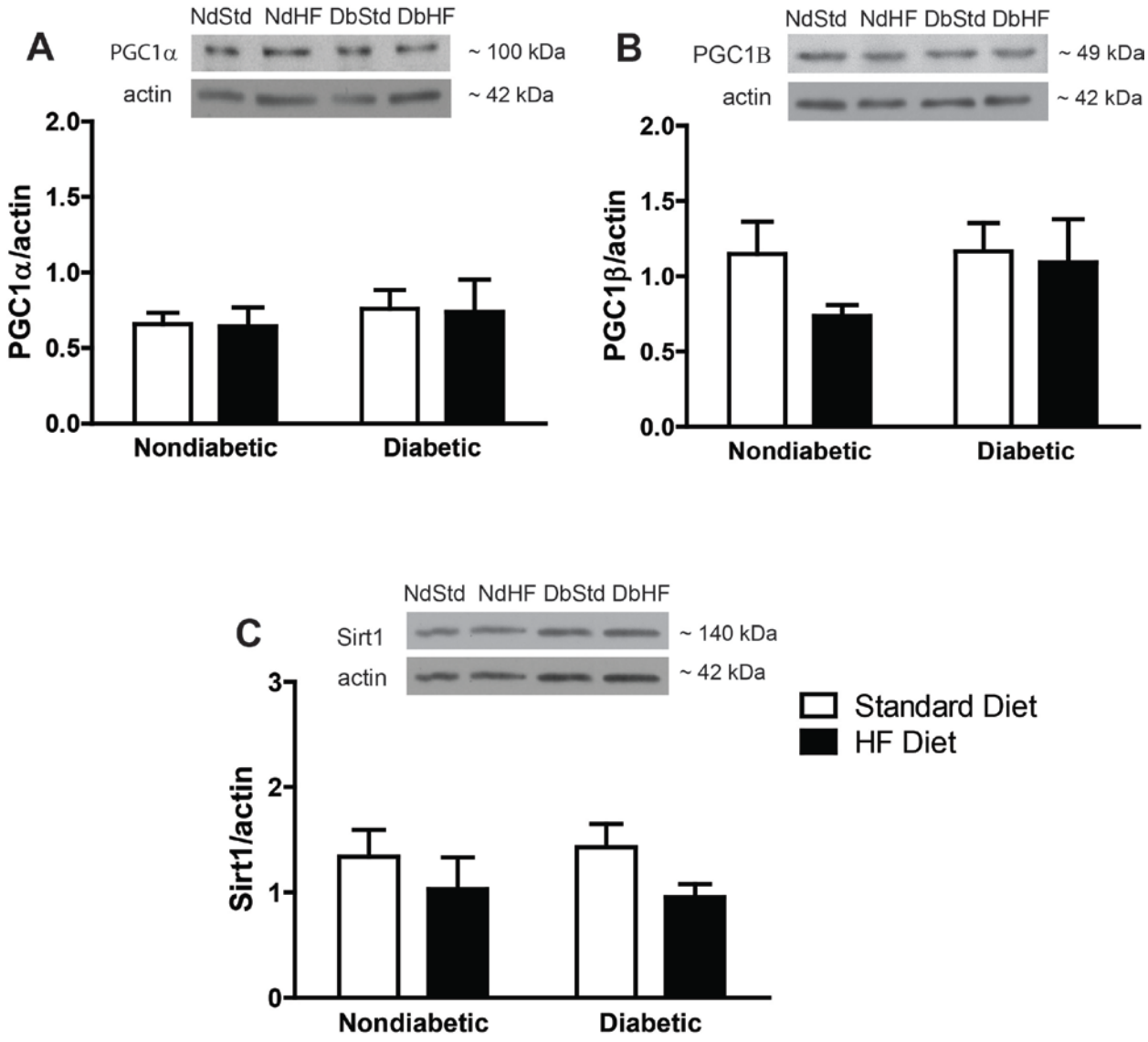

A
NdStd NdHF DbStd DbHF
PGC1α
~ 100 kDa
actin
~ 42 kDa
PGC1α/actin
2.0
1.5
1.0
0.5
0.0
Nondiabetic
Diabetic
B
NdStd NdHF DbStd DbHF
PGC1B
~ 49 kDa
actin
~ 42 kDa
PGC1β/actin
2.0
1.5
1.0
0.5
0.0
Nondiabetic
Diabetic
C
NdStd NdHF DbStd DbHF
Sirt1
~ 140 kDa
actin
~ 42 kDa
Sirt1/actin
3
2
1
0
Nondiabetic
Diabetic
Standard Diet
HF Diet

Figure 5: Expression of proteins involved in signaling pathways that affect mitochondrial density in the lumbar dorsal root ganglia.

Proteins were measured by Western blot. Tissues were harvested at 8 weeks post-STZ and high-fat diet. Representative images and quantification of group means for pAkt (A), pMtor, and (C) pAmpK. Band intensities for phosphorylated proteins were normalized to the total protein. Data are presented as means ± SEM (n = 8-10 mice per group). No significant differences among groups.

Figure 5

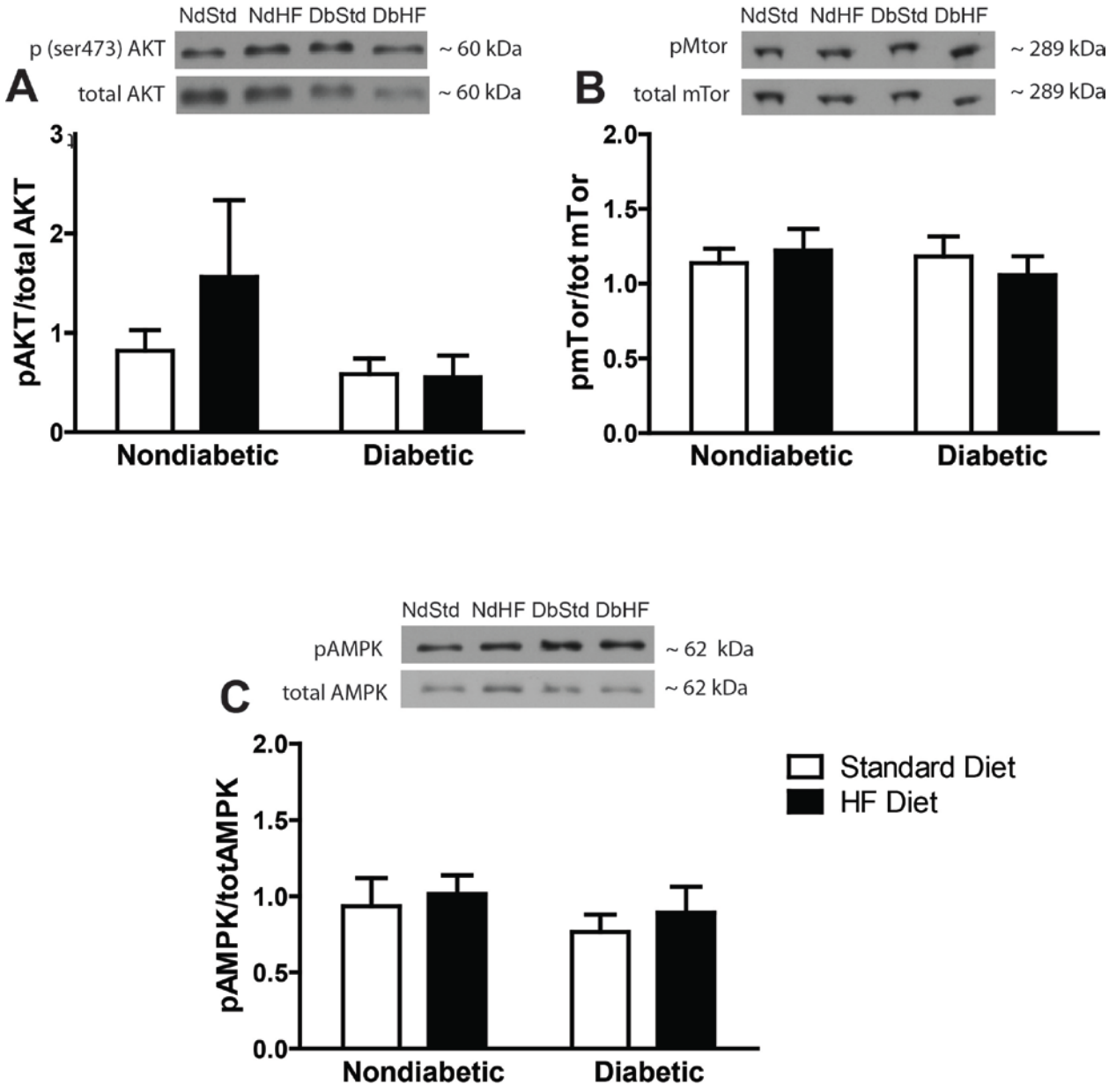
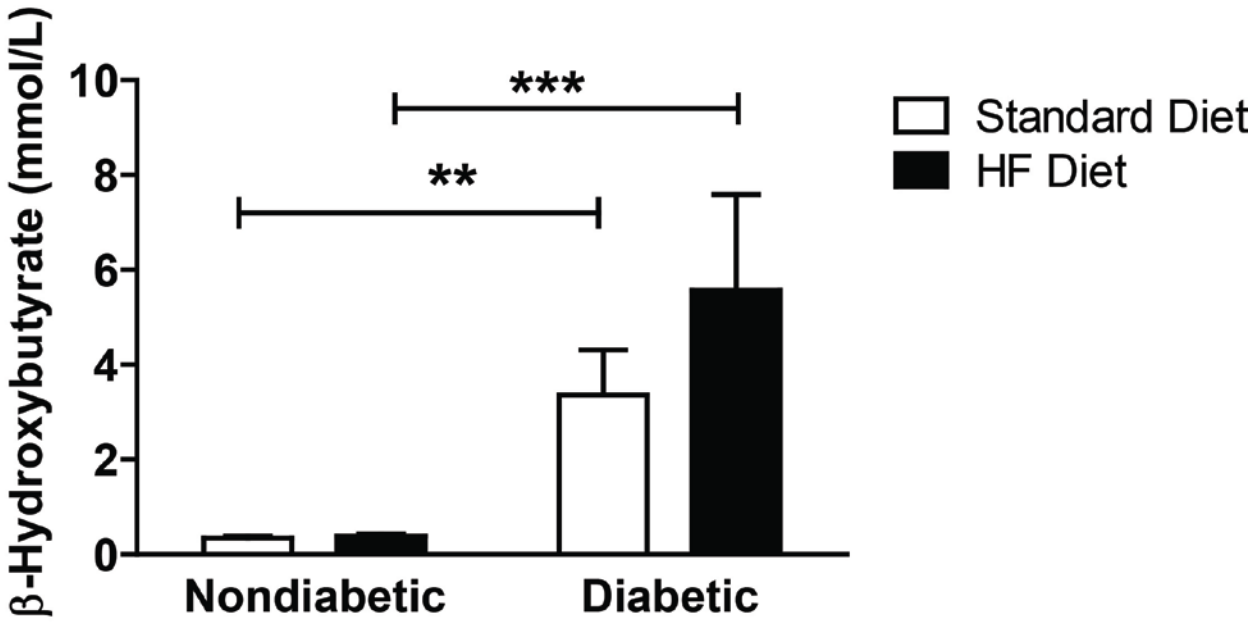

NdStd NdHF DbStd DbHF
p (ser473) AKT
~ 60 kDa
A
total AKT
~ 60 kDa
pAKT/total AKT
3
2
1
0
Nondiabetic
Diabetic
NdStd NdHF DbStd DbHF
pMtor
~ 289 kDa
B
total mTor
~ 289 kDa
pmTor/tot mTor
2.0
1.5
1.0
0.5
0.0
Nondiabetic
Diabetic
NdStd NdHF DbStd DbHF
pAMPK
~ 62 kDa
C
total AMPK
~ 62 kDa
pAMPK/totAMPK
2.0
1.5
1.0
0.5
0.0
Nondiabetic
Diabetic
Standard Diet
HF Diet

Figure 6: Serum β-hydroxybutyrate levels after 8 weeks of high-fat diet and/or diabetes.

Data are presented as means ± SEM (*n* = 10-18 mice per group). **P < 0.01, ***P < 0.001.

Figure 6


β-Hydroxybutyrate (mmol/L)
10
8
6
4
2
0
***
**
Nondiabetic
Diabetic
Standard Diet
HF Diet

indicate ketoacidosis and reduced blood pH, although these cut off values are not defined in rodents [214].

Because transient receptor potential cation channel subfamily V member 1 (TRPV1), an ion channel that has been implicated in chronic pain conditions [215], responds to a drop in pH or heat [216, 217], and high-fat fed mice exhibit mechanical hyperalgesia, we examined the relationship between β-HB levels and sensorimotor behavior after 8 weeks of diabetes and/or high-fat feeding (behavioral data was collected and described in Chapter 2). There were no significant correlations between β-HB levels and percent paw withdrawal (Figures 7A, 7C, 7E, mechanical sensitivity) in the NdHF, DbStd, or DbHF groups. However, increased withdrawal latency in response to a thermal stimulus was positively correlated β-HB levels (Figure 7D) in DbStd. β-HB levels were not correlated with thermal sensitivity in NdHF or DbHF (Figures 7B and 7F). NdHF mice do not display altered thermal sensitivity, thus we did not expect a significant correlation in this treatment group for this behavioral test (Figure 7B).

β-Hydroxybutyrate treated cells

Because both diabetic groups had significantly increased serum β-HB levels (Figure 6) and β-HB has been shown to affect mitochondrial respiration [218], the effect of β-HB treatment on expression of mitochondrial oxidative phosphorylation proteins and VDAC in cultured SY5Y neurons was examined. Eight-hour β-HB treatment had no effect on mitochondrial oxidative phosphorylation proteins (Figure 8A-D) or VDAC expression (Figure 8E) compared to untreated control cells.

Figure 7: Correlations between serum β-hydroxybutyrate level and behavioral signs of neuropathy.

Correlations between β-hydroxybutyrate level and percent paw withdrawal, a measure of mechanical sensitivity (A, C, E) and withdrawal latency, a measure of thermal sensitivity (B, D, F) in NdHF (A-B), DbStd (C-D), and DbHf (E-F). n = 8-18 mice per group for correlations with mechanical sensitivity (A, C, E) and n = 3-8 mice per group for correlations with thermal sensitivity (B, D, F). NdHF and DbHF exhibit mechanical hyperalgesia while DbStd mice exhibit mechanical insensitivity. DbStd and DbHF display thermal hypoalgesia. Thermal hypoalgesia was significantly correlated with β-hydroxybutyrate levels in DbStd: *P < 0.05.

Figure 7

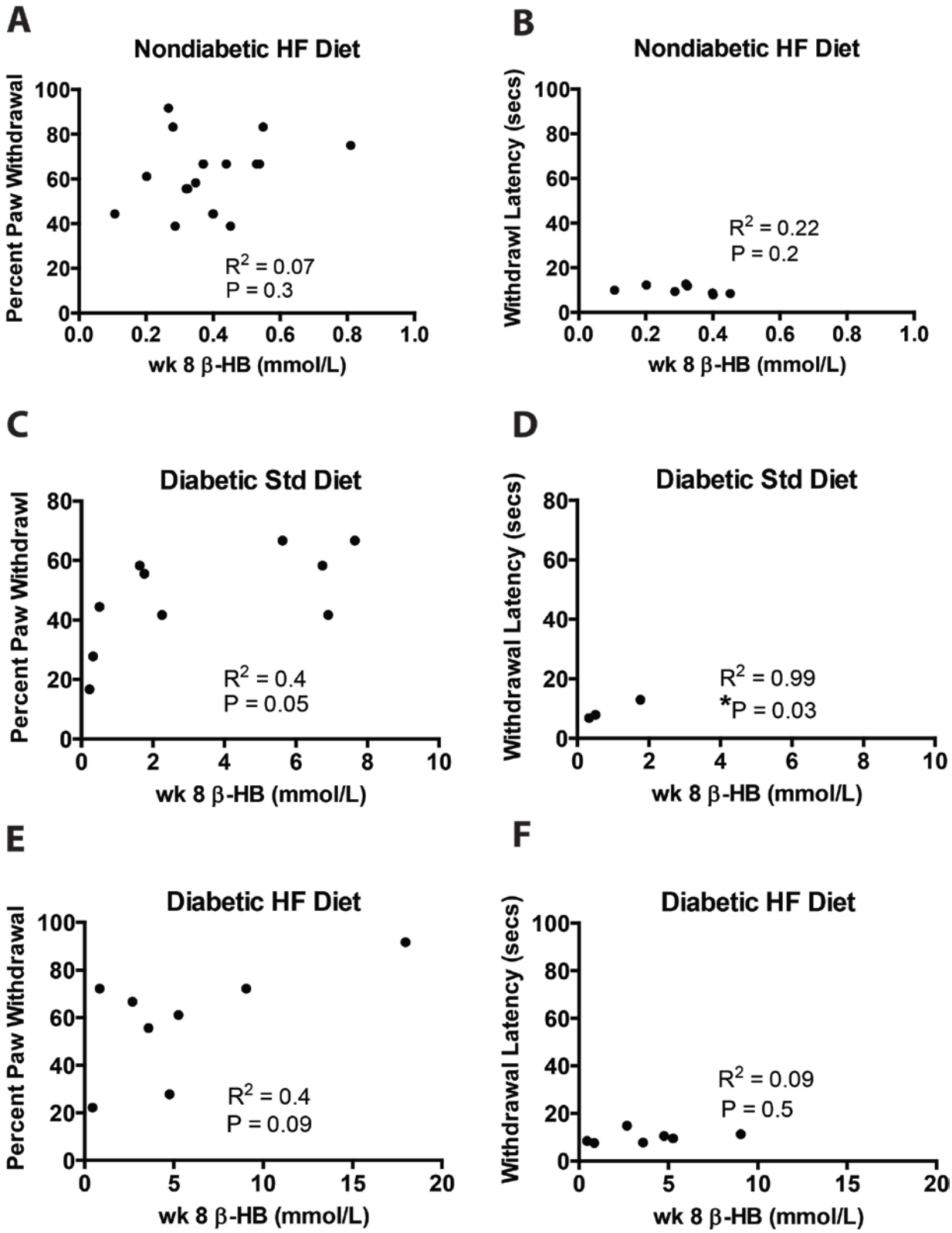
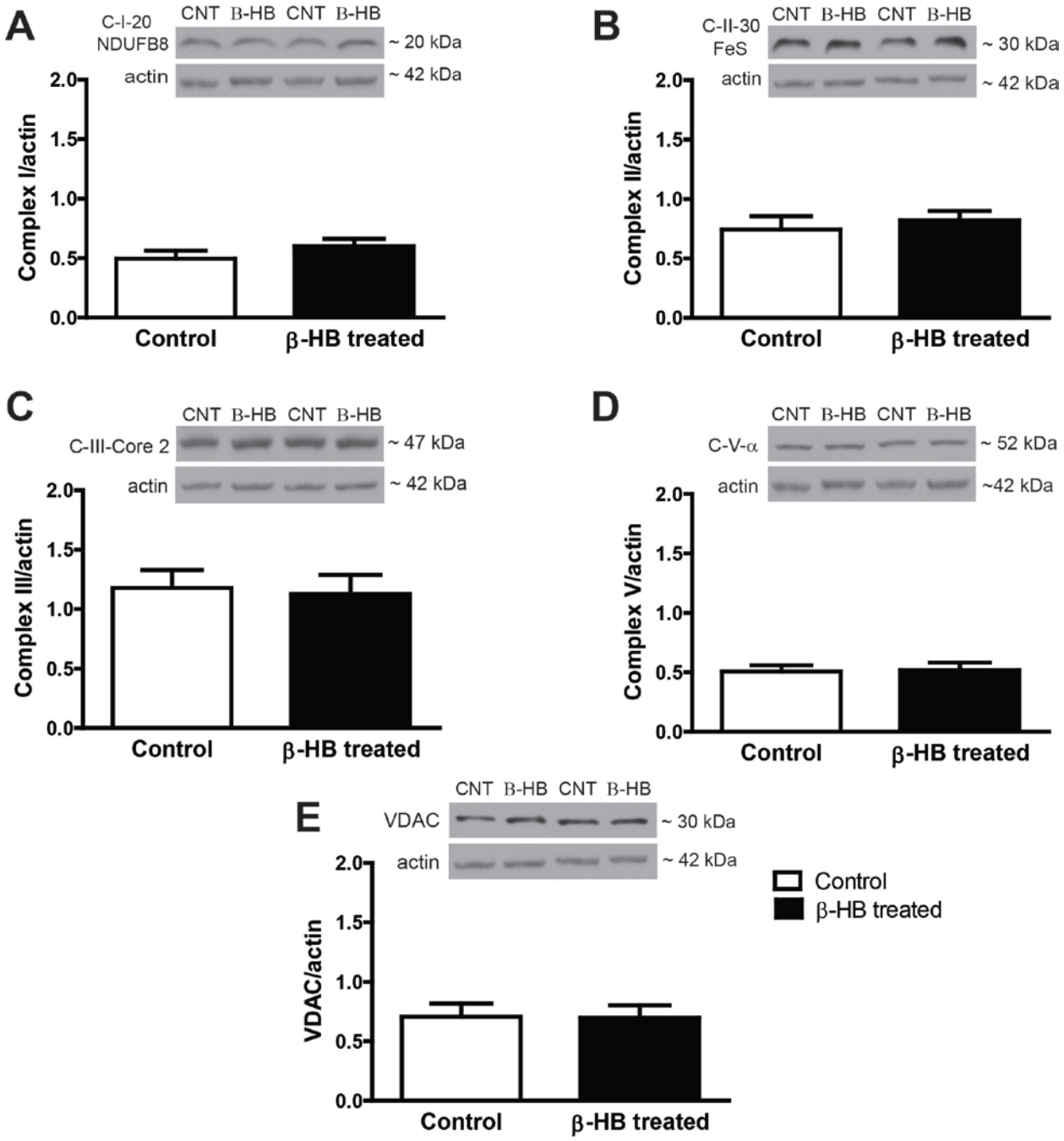

A
Nondiabetic HF Diet
Percent Paw Withdrawal
wk 8 β-HB (mmol/L)
R2 = 0.07
P = 0.3
B
Nondiabetic HF Diet
Withdrawl Latency (secs)
wk 8 β-HB (mmol/L)
R2 = 0.22
P = 0.2
C
Diabetic Std Diet
Percent Paw Withdrawl
wk 8 β-HB (mmol/L)
R2 = 0.4
P = 0.05
D
Diabetic Std Diet
Withdrawal Latency (secs)
wk 8 β-HB (mmol/L)
R2 = 0.99
*P = 0.03
E
Diabetic HF Diet
Percent Paw Withdrawal
wk 8 β-HB (mmol/L)
R2 = 0.4
P = 0.09
F
Diabetic HF Diet
Withdrawal Latency (secs)
wk 8 β-HB (mmol/L)
R2 = 0.09
P = 0.5

Figure 8: Expression of mitochondrial proteins in control and β-hydroxybutyrate treated SH-SY5Y neuronal cells.

Cells were harvested after 8 hour treatment in DMEM supplemented with 5 mM glucose (control) or 5 mM glucose and 5 mM β-hydroxybutyrate (β-HB treated). Proteins were measured by Western blot. Representative images and quantification of group means for subunits of mitochondrial oxidative phosphorylation complexes: A) complex I, B) complex II, C) complex III, and D) complex V, and VDAC, a mitochondrial protein that is not involved in oxidative phosphorylation (E). Data are presented as means ± SEM (n = 5 cell lysates per group). No significant differences.

Figure 8


A
C-I-20
NDUFB8
actin
CNT B-HB CNT B-HB
~ 20 kDa
~ 42 kDa
Complex I/actin
2.0
1.5
1.0
0.5
0.0
Control
β-HB treated
B
C-II-30
FeS
actin
CNT B-HB CNT B-HB
~ 30 kDa
~ 42 kDa
Complex II/actin
Control
β-HB treated
C
C-III-Core 2
actin
CNT B-HB CNT B-HB
~ 47 kDa
~ 42 kDa
Complex III/actin
Control
β-HB treated
D
C-V-α
actin
CNT B-HB CNT B-HB
~ 52 kDa
~42 kDa
Complex V/actin
Control
β-HB treated
E
VDAC
actin
CNT B-HB CNT B-HB
~ 30 kDa
~ 42 kDa
VDAC/actin
Control
β-HB treated
Control
β-HB treated

5. Discussion

Recent evidence suggests mitochondrial dysfunction may play an important role in the pathogenesis underlying diabetic neuropathy . Here, for the first time, we report mitochondrial respiration data collected from undisrupted freshly isolated lumbar DRG and peripheral axonal processes. All previous reports documenting mitochondrial dysfunction in animal models of diabetic neuropathy have performed experiments on primary DRG cultures, isolated mitochondria, or DRG homogenates from diabetic animals [20, 22, 63, 64, 66, 68, 73]. In effort to improve upon these experiments and more closely emulate the *in vivo* environment, we measured mitochondrial respiration in whole tissue (lumbar DRG and peripheral axonal processes) immediately following dissection. In addition, for the first time, mitochondrial respiration was assessed in the DRG and axonal processes in high-fat fed mice. The use of a high-fat diet in conjunction with type I diabetes is a novel, clinically relevant model of diabetic neuropathy and previous studies of mitochondrial dysfunction in diabetic neuropathy have not incorporated diet into mitochondrial assessments.

Diabetes Impairs Some Aspects of Mitochondrial Respiration and Alters the Mitochondrial Proteome

Our results reveal that after 8 weeks of diabetes, basal mitochondrial respiration was decreased in the lumbar DRG and peripheral axonal processes from diabetic mice fed a standard diet compared to their nondiabetic counterparts. Importantly, results from our experiments using

this novel methodology are consistent with previous studies that report mitochondrial respiration deficits in DRG from diabetic mice [20, 63, 64, 66].

In contrast, spare respiratory capacity and expression of mitochondrial proteins VDAC and Complex III subunit Core-2 were increased in diabetic mice fed the standard diet compared to nondiabetic mice fed the standard diet. VDAC is an ion channel protein that is abundantly present on the mitochondrial membrane [219]. Thus, increased VDAC expression may indicate increased mitochondrial mass. Complex III subunit Core-2 is a subunit of the mitochondrial oxidative phosphorylation Complex III. Increased expression of specific mitochondrial oxidative phosphorylation proteins suggest increased mitochondrial mass or an isolated increase in a specific oxidative phosphorylation protein in effort to preserve respiration despite mitochondrial damage. These results oppose data from several previous studies that indicate mitochondrial oxidative phosphorylation protein expression is reduced in DRG from diabetic rodents [63, 64, 66, 220]. However, the current study was only 8 weeks in duration and mitochondrial function was in examined in mice rather than rats.

Spare respiratory capacity is calculated by subtracting the maximal mitochondrial oxygen flux rate from the basal mitochondrial oxygen flux rate. Spare respiratory capacity represents the capability of mitochondria to increase respiration beyond basal respiration. Typically, damaged mitochondria exhibit reduced maximal respiration and/or reduced spare respiratory capacity. Therefore, it is surprising that diabetic mice fed the standard diet had significantly higher spare respiratory capacity compared to nondiabetic mice fed the standard diet. However, it is important to note that in all groups except NdStd, spare respiratory capacity was negative, indicating maximal respiration was lower than basal respiration.

When the experimental conditions and drug concentrations are optimized, the maximal oxygen flux rate is usually greater than or equal to the basal oxygen flux rate in control tissue (NdStd in this case), resulting in zero or positive spare respiratory capacity. A noteworthy caveat is the fact that spare respiratory capacity was positive in the majority of DbStd mice while spare respiratory capacity was negative in the majority of the mice from the other three groups. Although careful efforts and numerous pilot experiments were performed to optimize the assay conditions for whole undisrupted tissue, it is possible the FCCP concentration was not potent enough to elicit maximal respiration. Because the diabetic animals fed the standard diet are lower in body weight and have smaller tissue volume, the FCCP potentially had a more potent effect on the DbStd tissue, while the FCCP injection had a less potent effect on the typically larger tissue of animals from the NdStd, NdHF, or DbHF groups. This caveat introduces uncertainty, suggesting that true maximal respiration was not achieved in experiments in animals from all groups, thus deeming the maximal oxygen flux rate and spare respiratory capacity data inconclusive. If the FCCP potency were sufficient to stimulate maximal respiration in variable tissue volumes from animals in all groups, perhaps the spare respiratory capacity would be diminished in DbStd compared to NdStd.

In the current study, mitochondrial respiration was normalized to total protein to correct for differences in tissue volume and subsequently normalized to VDAC expression to account for mitochondrial density. When the VDAC normalization was removed and mitochondrial respiration was normalized to total protein only, there were no significant differences among groups in basal mitochondrial respiration (NdStd ± 191.5, NdHF 196.3 ± 24.6, DbStd 193.5 ± 18.1, DbHF 172.3 ± 12.8 pmols O_2 sec^{-1} mg $protein^{-1}$). This suggests that the total amount of basal mitochondrial respiration occurring in the tissue is not altered in diabetic mice, but the

amount of respiration being performed by each mitochondrion may be diminished. Reduced basal mitochondrial respiration suggests diabetes induced mitochondrial stress while increased VDAC and Complex III subunit Core-2 expression indicates potential mitochondrial biogenesis. Together, these results suggest that diabetes negatively impacts mitochondrial function and compensatory mitochondrial biogenesis may occur to maintain mitochondrial respiration.

The high-fat diet did not appear to significantly impact mitochondrial function in nondiabetic or diabetic mice as there were no other differences among other groups in mitochondrial respiration or mitochondrial protein expression. The effects of high-fat feeding on mitochondrial function in sensory neurons have not been documented in the literature.

Expression of Proteins Related to Mitochondrial Biogenesis and Mitochondrial Function are Not Altered by Diabetes or a High-Fat Diet

Diabetes and high-fat feeding have each been purported to alter expression of other proteins that play an important role in mitochondrial function and/or mitochondrial biogenesis. In this study, neither diabetes nor the high-fat diet had an effect on UCP2, PGC-1α, PGC1β, or Sirt 1 expression, or Akt, AMPK, or mTor activation in the lumbar DRG and peripheral axonal processes.

UCP2 is a mitochondrial uncoupling protein that resides in the inner mitochondrial membrane, facilitates proton return from the outer to inner mitochondrial membrane, and separates oxidative phosphorylation from ATP synthesis while dissipating energy as heat [221]. Increased UCP2 expression can cause increased proton leak from the outside into the mitochondria and may contribute to increased leak dependent respiration. However, since there

were no differences among groups in leak dependent respiration, it is not surprising that UCP2 expression was not affected by diabetes or the high-fat diet.

Because changes in mitochondrial respiration and the mitochondrial proteome suggest that diabetes stimulated mitochondrial biogenesis in mice fed the standard diet, we investigated the effect of diabetes and the high-fat diet on expression of proteins that play an important role in mitochondrial biogenesis and autophagy. Figure 9 illustrates the relationships between PGC-1α, Sirt 1, Akt, and mTor with notable convergence on PGC-1α activation, mitochondrial biogenesis, and autophagy.

PGC-1α and PGC-1β belong to a family of transcription coactivators that play a central role in the regulation of cellular energy metabolism [222]. PGC-1α interacts with transcription factors and/or nuclear receptors to stimulate mitochondrial biogenesis, hepatic gluconeogenesis, adaptive thermogenesis, and/or fatty acid and glucose metabolism [222]. Although the functions of PGC-1β are not as well documented as PGC-1α, its functions are similar and participates with PGC-1α in coordinated coactivation of metabolically relevant transcription factors to stimulate mitochondrial biogenesis and fatty acid oxidation. PGC-1α and PGC-1β function is compromised in obesity, insulin resistance, and type 2 diabetes [223]. AMPK acts as an energy sensor and key regulator of cellular energy homeostasis by responding to an increased AMP/ATP ratio. AMPK is activated by phosphorylation when ATP is depleted and among other functions, phosphorylated AMPK activates PGC-1α [224]. The AMPK/ PGC-1α signaling axis is decreased in hyperglycemia and recent evidence suggests that neuronal dysfunction in diabetic neuropathy is associated with mitochondrial dysfunction due to impaired AMPK/ PGC-1α signaling [224]. Chowdhury et al. reported reduced PGC-1α expression and reduced AMPK

Figure 9

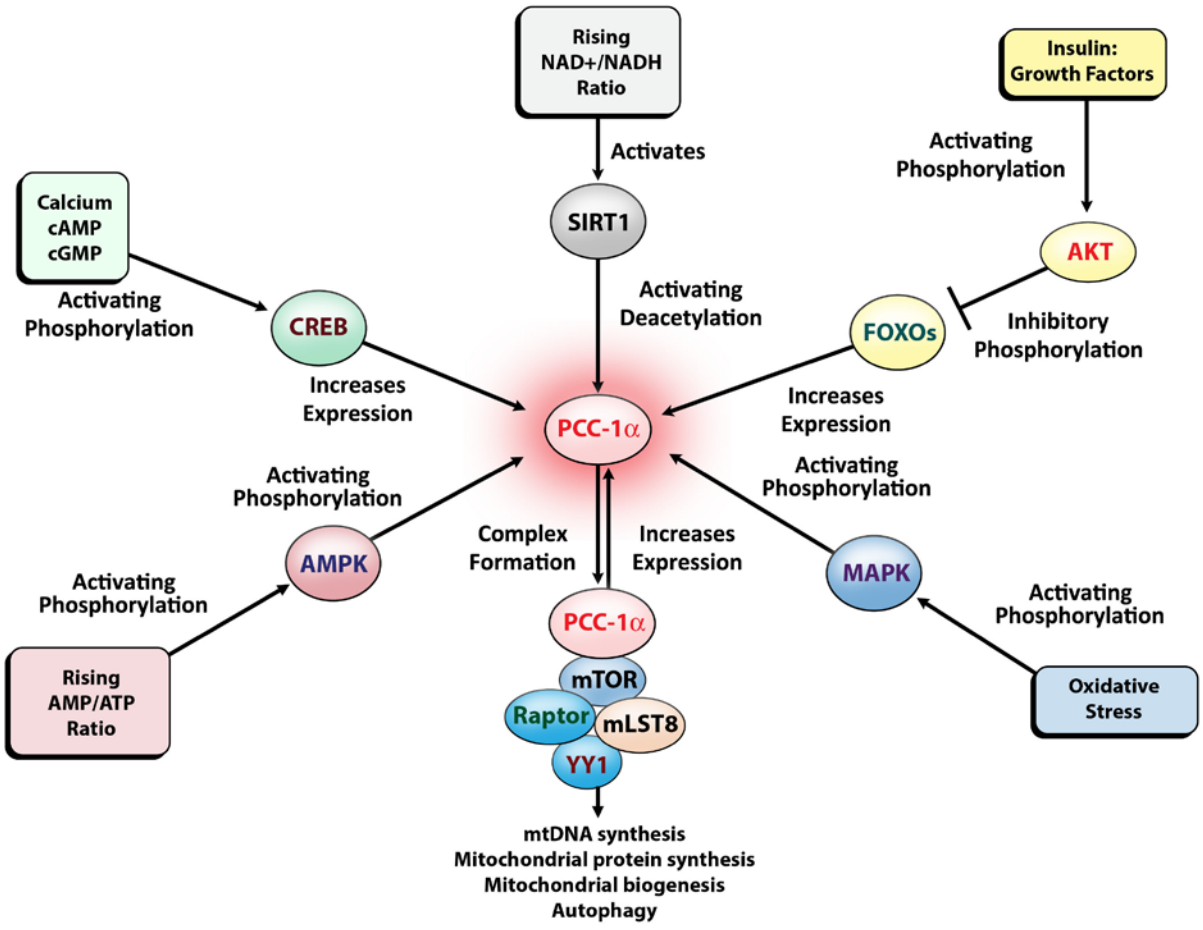


Figure 9: Schematic illustrating activation and inhibition of proteins that activate PGC-1α.

This content in this figure was developed by Russell Swerdlow and creatively enhanced by Stanton Fernald.

activation in DRG from STZ-induced diabetic rats and *db/db* mice [64]. However, it is important to note that in Chowdhury et al., there were no changes in AMPK activation or PGC-1α after 4 weeks of diabetes, and deficits in these proteins were not seen until after 8 weeks of diabetes [64]. In the current study, there were no differences among groups in PGC-1α and PGC-1β expression or AMPK activation in the DRG after 8 weeks of diabetes. In addition, reduced basal mitochondrial respiration accompanied by increased mitochondrial proteins in diabetic mice fed the standard diet suggested potential compensatory mitochondrial biogenesis. Thus its plausible to expect increased PGC-1α expression in DbStd compared to NdStd. Perhaps these tissues were assessed on the cusp of a time point when subtle changes begin to appear in these proteins and any changes were too subtle to be detected by Western blot at this time point. Alternatively, PGC-1α expression may have increased earlier in time course of diabetes in order to trigger compensatory mitochondrial biogenesis and may have returned to normal levels by the time tissues were harvested after 8 weeks of diabetes.

Sirt 1 is a nutrient-sensing histone deacetylase that is activated by a rising NAD^+/NADH ratio and plays an important role in regulating metabolic function in many tissues [225]. In adipose tissue, Sirt 1 protects from obesity and inflammation under normal feeding conditions [225], is a positive regulator of insulin secretion [226] and autophagy [227], and has been identified as a potential therapeutic target for type 2 diabetes and diabetic complications [228]. Sirt 1 deacetylates, and thereby activates PGC-1α and indirectly stimulates mitochondrial biogenesis [229]. Sirt 1 is expression and activity is reduced in liver adipose tissue of high-fat fed rodents [225]. Furthermore, a Sirt1 genetic variant is associated with reduced insulin sensitivity and is a type 2 diabetes risk in Pima Indians [230]. Although Sirt-1 expression was slightly reduced in the DRG of high-fat fed nondiabetic and diabetic mice in the current study,

these differences did not reach statistical significance in this cohort of animals. If diabetes and high-fat feeding were greater than 8 weeks in duration, significant changes in Sirt-1 expression may have been apparent.

Akt is a serine/threonine kinase that plays a pivotal role in several cellular processes including cell proliferation and migration, apoptosis, and glucose metabolism. Akt is a downstream effector of PI 3-kinsases and is a key protein in the insulin-signaling pathway in peripheral tissues and neurons [231, 232]. The active form of Akt is phosphorylated (pAkt) on serine reside 473, and Akt activation is reduced in insulin resistant states [132]. In fact, insulin-stimulated Akt activation is blunted in DRG neurons from diabetic mice [132]. Insulin signaling, via downstream Akt activation is important for maintenance of mitochondrial function in neurons [21, 66]. Because high-fat fed nondiabetic mice exhibited hyperinsulinemia and hyperglycemia in the current study, we suspected these mice were mildly insulin resistant and hypothesized Akt activation might be blunted in the DRG. There were no significant differences among groups in Akt activation in the DRG. However, Grote and colleagues [132] found that Akt activation was assessed in primary cultured DRG after insulin stimulation while there was no insulin stimulation and the DRG were not cultured in the current study.

mTor is a serine/threonine protein kinase that responds to nutritional status, growth factor and stress signals, and plays a key role at the interface of pathways that regulate the balance between cell growth and autophagy . When activated, mTor associates with other proteins and can be present in one of two complexes (mTORC1 or mTORC2). Nutrient starvation or reduced growth factor availability can activate mTor and mTORC1 complex formation, and subsequently induce autophagy [233]. Akt is upstream and positively regulates mTor while active AMPK inhibits mTORC1. Because mTORC1 is inhibited by oxidative stress and mitochondrial

dysfunction, it has been suggested that mTORC1 may be involved in the mechanism by which damaged mitochondria induce autophagy (9, 44). In the present study, it is possible that mTor activation was altered by nutrient starvation in diabetic mice fed the standard diet or by the downstream effects of insulin resistance in nondiabetic high-fat fed mice. Based on the nutritional status of these groups of animals, we might expect increased mTor activation and increased autophagy in DbStd and/or reduced mTor activation and reduced autophagy in NdHF. However, increased mitochondrial proteins in diabetic mice fed the standard diet would not be congruent with increased autophagy, unless it was accompanied by mitochondrial biogenesis. In the current study, there were no differences among groups in phosphor mTor expression, thus the high-fat diet, nor diabetes impacted mTor activation.

Increased β-Hydroxybutyrate Levels are Not Correlated with Mechanical Hyperalgesia

According to cut-off values for human ketoacidois and previous studies in rodents, both diabetic groups exhibited increased serum *β*-HB levels, indicative of diabetic ketoacidosis [214]. Because blood pH is lowered in the ketoacidotic state [214] and TRPV1, an ion channel that has been implicated in chronic pain states responds to reductions in pH [215], we hypothesized increased *β*-HB levels might be correlated with mechanical hyperalgesia in the NdHF and DbHF group. However, *β*-HB levels were not significantly increased in NdHF, nor was *β*-HB correlated with mechanical hyperalgesia in either group. Although we did not expect *β*-HB levels to be correlated with thermal hypoalgesia, withdrawal latency was positively correlated with *β*-HB levels in DbStd but not DbHF. However, specific to the *β*-HB correlation with thermal sensitivity for DbStd, there were only three mice in the DbStd group. Therefore, the significant correlation between *β*-HB level and thermal hypoalgesia in the DbStd group should be

interpreted with caution. Mice in the DbHF group also exhibit thermal hypoalgesia and elevated *β*-HB, but there was not a significant correlation between these variables in the DbHF group (p = 0.09). However, the P-value suggests a trend toward a positive correlation between withdrawal latency and *β*-HB in DbHF, would is consistent with the effect as observed in DbStd. Although DbStd and DbHF exhibit opposite behavioral responses to a mechanical stimulus (insensitivity in DbStd and hyperalgesia in DbHF), both groups display thermal hypoalgesia. We expect that *β*-HB level is positively correlated with withdrawal latency due to severity of diabetes or hyperglycemia. DbStd mice had consistently higher glucose levels than DbHF.

β-Hydroxybutyrate Treatment Does Not Alter Expression of Mitochondrial Proteins in Cultured Neurons

Recent evidence indicates circulating ketones can impact mitochondrial respiration, mitochondrial biogenesis, and the mitochondrial proteome [218, 234]. We hypothesized that ketosis may alter mitochondrial respiration and protein expression in neurons. Consequently, we performed an experiment on cultured SY5Y neurons to determine if treatment with β-HB altered mitochondrial protein expression. There were no significant differences in expression of VDAC or subunits of mitochondrial oxidative phosphorylation complexes in control versus 8 hour β-HB treated cells. Perhaps the result would be different had we used primary cultures from nondiabetic and diabetic mice or if β-HB exposure was chronic rather than acute. Although both diabetic groups had elevated β-HB compared to their nondiabetic counterparts, the high-fat diet did not exacerbate ketosis.

Summary and Conclusions

In summary, diabetes significantly reduced basal mitochondrial respiration with a concomitant increase in VDAC and Complex III subunit Core-2 expression in DRG and peripheral axonal processes isolated from mice fed a standard diet. Surprisingly, were no changes in mitochondrial respiration or mitochondrial protein expression in high-fat fed nondiabetic or diabetic mice. If altered mitochondrial function in diabetic mice is primarily mediated by hyperglycemia, diabetic mice fed the high-fat diet may be partially protected from these effects via less severe hyperglycemia resulting from lower dietary carbohydrate content compared to diabetic mice fed the standard diet.

Taken together, these data suggest subtle mitochondrial dysfunction and potential compensatory mitochondrial biogenesis occurs after 8 weeks of STZ-induced diabetes and high-fat feeding does not alter these parameters. These data support previous results that indicate diabetes-induced deficits in mitochondrial respiration. This work has a significant impact on the diabetic neuropathy field as it employed novel methodology by measuring mitochondrial respiration in freshly isolated undisrupted DRG and peripheral axonal processes. In addition, for the first time, mitochondrial function was assessed in STZ-induced type 1 diabetic mice fed a high-fat diet. In light of the recent clinical evidence identifying dyslipidemia as an independent risk factor for the development of diabetic neuropathy, this type 1 diabetic/high-fat combination is a novel and clinically relevant model that is useful for studying diabetic neuropathy. Because mitochondrial dysfunction is subtle in the DRG and significant mitochondrial impairment may also occur in axonal transport and trafficking, future studies should investigate the effect of diabetes on mitochondrial function in peripheral nerves responsible for innervating the lower extremities.

CHAPTER 4

The Role of Inflammation in High-Fat Diet-Induced Hyperalgesia

1. Abstract

Previous data reveals that a high-fat diet fed to STZ-induced diabetic C57BL/6 mice strongly alters the neuropathy phenotype by inducing mechanical hyperalgesia instead of insensitivity that normally develops in this model. HF fed nondiabetic mice also develop mechanical hyperalgesia. Pro-inflammatory cytokines and chemokines contribute to the development of nociception, including diabetic neuropathy. A high-fat diet induces pro-inflammatory cytokine production, suggesting diet-induced inflammation may drive mechanical hyperalgesia. Additionally, increased cytokine and chemokine levels are associated with glial activation in the spinal cord, suggesting neural inflammation and glial activation are important central nervous system mediators of pain. The purpose of this study was to determine if a high-fat diet increases induces inflammation in the spinal cord or peripheral tissues. We hypothesized that a high-fat diet induces central and/or peripheral inflammation in conjunction with glial activation in the spinal cord, thus resulting in chronic mechanical hyperalgesia. Following STZ or vehicle injection, male C57Bl/6 mice were fed a standard or high-fat diet for 8 weeks. Consistent with previous findings in this insensate model of diabetic neuropathy, diabetic mice fed the standard diet exhibited mechanical insensitivity compared to nondiabetic mice fed the standard diet. In contrast, high-fat-fed nondiabetic and diabetic mice developed robust mechanical hyperalgesia. Surprisingly, there were no significant differences among groups in cytokines or chemokines: Il-1α, Il-1β, Il-2, TGFβ, Il-10, Il-12p70, Il-17, MCP-1, or TNF-α in the lumbar spinal cord. Furthermore, microglial activation was not increased by diabetes or a high-fat diet. Although TNF-α levels were not altered in peripheral tissues, serum TNF-α levels were lower in high-fat-fed nondiabetic mice and standard-fed diabetic mice compared to nondiabetic mice fed the standard diet. In conclusion, high-fat diet-induced mechanical hyperalgesia was not

accompanied by spinal or peripheral inflammation or microglial activation after 8 weeks of high-fat feeding. Although inflammation was not apparent at week 8, it is plausible that the inflammatory response occurred at an earlier time point near the initiation of mechanical hyperalgesia. Therefore, future experiments will examine spinal and peripheral inflammation after 4 weeks of high-fat feeding which is near the onset of behavioral changes in mechanical sensitivity.

2. Introduction

There are a number of proposed mechanisms that contribute to diabetic neuropathy but no information is available as to why diabetic patients either develop painful symptoms (painful neuropathy) or a loss of sensation (insensate neuropathy). Emerging studies now suggest that spinal cord inflammation plays a key role in chronic pain. Increased pro-inflammatory cytokine and chemokine synthesis is associated with glial activation in the spinal cord, suggesting that neural inflammation and glial activation may collaboratively increase nociception and lead to chronic pain.

It has been well documented that STZ-induced diabetic C57Bl/6 mice develop a moderate insensate neuropathy characterized by a mild reduction in mechanical sensitivity after 4 weeks of diabetes [181-183, 189, 235]. In striking contrast, STZ-diabetic mice fed a high-fat diet display a painful diabetic neuropathy phenotype as opposed to the insensate phenotype typically observed in this strain (Reviewed in Ch.2 [30]). Interestingly, the high-fat diet induced a robust mechanical hyperalgesia; mechanical sensitivity was increased by 35% and 45%, respectively, in nondiabetic and diabetic mice compared to their counterparts fed a standard diet (Reviewed in Ch.2 [30]). This finding is particularly important because it suggests that diet modulates diabetic neuropathy phenotype in rodents and may shed light on why some patients experience insensate symptoms while others have painful symptoms. In addition, this data supports previous reports that a high-fat diet can induce neuropathy in nondiabetic mice [5, 23].

Little is known about mechanisms contributing to high-fat diet-induced painful neuropathy, but a recent study indicates that an extract from the Artemisia plant (known for its anti-inflammatory and anti-nociceptive properties) alleviates high-fat diet induced mechanical hyperalgesia and reduces 12/15 lipoxygenase (regulates pro-inflammatory cytokine production)

upregulation, suggesting that inflammation may play a role in high-fat diet induced neuropathy [190]. Pro-inflammatory cytokines and chemokines have been widely implicated in chronic pain and are thought to contribute to the central sensitization that results in mechanical hyperalgesia [198-201]. For example, interleukin-6 (IL-6) via its actions in the dorsal horn induces allodynia and hyperalgesia [236], and interleukin 1-beta (IL1-β) enhances the spinal release of substance P [237]. In addition, inflammation can induce a phenotypic switch in A-β fibers so that they acquire properties of pain-sensing C-fibers, thus enhancing spinal synaptic transmission and contributing to hypersensitivity [199].

In rodent models of neuropathic pain, increased pro-inflammatory cytokine and chemokine synthesis is associated with glial activation in the spinal cord, suggesting that neural inflammation and glial activation are important mediators within central nervous system associated with pain [238-242]. Activated glia are abundant sources of pro-inflammatory cytokines and can modify synaptic transmission and nociception via interactions with neurons [206-210]. In addition, glial activation has been shown to be necessary and sufficient to create pathological pain in laboratory animals [243-245] and microglia are activated in the spinal dorsal horn of STZ-induced diabetic rats that display mechanical hyperalgesia [246].

Importantly, obesity is associated with chronic low-grade inflammation [79, 80], and a high-fat diet increases the pro-inflammatory cytokines IL1-β, tumor necrosis factor alpha (TNF-α), and IL1-6, and stimulates inflammatory signaling in adipose, serum, liver, and brain [124, 202-205]. Along with glia and macrophages [206-211], adipose is an important source of pro-inflammatory cytokines that may upregulate cytokine production due to increased adipose resulting from a high-fat diet [124, 202].

Although high-fat diet induced neuropathy has been reported in the literature, only Obrosova et al. has investigated potential mechanisms underlying this phenomenon and suggests inflammation may play a role in high-fat diet induced mechanical hyperalgesia [190]. We initially postulated mitochondrial dysfunction might contribute to high-fat diet induced mechanical hyperalgesia, but results from our previous study (reviewed in Chapter 3) suggest mitochondrial dysfunction is likely not a key mechanism underlying diet-induced behavioral changes in nondiabetic or diabetic mice. Thus, additional investigation is needed to identify putative cellular changes associated with high-fat diet induced neuropathy in effort to identify potential mechanisms. Here, we report the effects of a high-fat diet on inflammatory factors in the spinal cord and peripheral tissues and microglial activation in the spinal cord in nondiabetic and diabetic mice.

3. Experimental Procedures

Animals and Diet

In this study, animal age, housing conditions and diet composition were the same as described in Chapters 2 and 3. Seven week-old male C57Bl/6 mice were purchased from Charles River (Wilmington, MA), housed two mice per cage under pathogen free conditions, and placed on a 12:12h light/dark cycle in the research support facility at the University of Kansas Medical Center. All animals had ad libitum access to food and water and were fed a standard diet (14% kcals from fat) or high-fat diet (54% kcals from fat). Animals in the high-fat diet group were fed the standard diet for 1 week prior to STZ-injection and 1 week after STZ-injection. The high-fat groups began the high-fat diet 1 week after STZ-injection. It is noteworthy to mention that mice

began the high-fat diet 3 hours post STZ-injection in experiments described in Chapters 2 and 3 rather than 1 week post STZ-injection. We delayed high-fat diet initiation to 1 week post-STZ in effort to cause less metabolic stress and reduce mortality in the diabetic high-fat group. All protocols and procedures were approved by the University of Kansas Medical Center Animal Use and Care Committee.

Diabetes Induction and Glucose Measurement

The diabetes induction, body weight, and glucose measurement protocols used in these experiments were identical to the protocols described in Chapters 2 and 3. STZ-injection was used to induce diabetes in 8-week old male C57Bl/6 mice. Nondiabetic mice were injected with vehicle buffer. All mice were fasted for 3 hours pre and post injection. Body weight and blood glucose were monitored weekly and criteria for inclusion in the diabetic groups were defined as described in Chapters 2 and 3. Treatment groups are abbreviated throughout as follows: nondiabetic standard diet (NdStd); nondiabetic high-fat diet (NdHF); diabetic standard diet (DbStd); and diabetic high-fat diet (DbHF).

Behavioral Test for Mechanical Sensitivity

Mechanical sensitivity was assessed at baseline (wk 0, immediately before STZ-injection) and every other week thereafter using the same procedures as described in Chapters 2 and 3. Mice were acclimated to the testing apparatus before baseline testing. Subsequently, mice were placed on an elevated wire mesh screen, enclosed individually in a small clear plastic cage, and allowed to acclimate on the table for 30 minutes before the test. Mechanical sensitivity was

assessed using 12 applications of 1.4 gram Semmes-Weinstein von Frey monofilament to the hindpaw footpads. The total of 12 applications was used to calculate mean percent withdrawal per mouse and used to calculate group means for each testing week.

Protein Extraction and Quantification of Cytokines and Chemokines

Cytokines and chemokines most frequently associated with inflammatory pain and excess dietary fat intake were quantified in the lumbar spinal cord and peripheral tissues. At 8 weeks post-STZ (or vehicle) injection, mice were anesthetized with isofluorane and decapitated. Lumbar spinal cord, sciatic nerves, gastrocnemius, and hindpaw plantar skin were dissected, snap frozen in liquid nitrogen, and stored at -80^0 C until protein extraction was performed. Blood was collected from the decapitation pool, clotted for 30 minutes on ice, and centrifuged for 15 minutes at 3,000 x *g*. Serum was removed and stored at -80^{o}C until analyzed for TNF-α. Lumbar spinal cord was homogenized with cell lysis buffer containing PBS at pH 7.4, 2 mM PMSF, and 1 µg/ml of each protease inhibitor (aprotinin, antipain, leupeptin, and pepstatin A). Sciatic nerves were sonicated and gastrocnemius and hindpaw plantar skin were homogenized with cell lysis buffer (137 mM NaCl, 20 mM Tris [pH 8.0], 1% NP40, and 10% glycerol) containing protease inhibitors (0.5 mm sodium vanadate, 1 µg/ml leupeptin, 10 µg/ml aprotinin, and 1 mM PMSF). Following homogenization, protein was extracted while tissue lysates were kept on ice for 1 hour and vortexed every 10 minutes. Samples were then centrifuged at 10,000 *g* for 10 minutes at 4^0 C and the protein concentration of the supernatant was measured using the Bio-Rad protein assay based on the Bradford method (Bio-Rad, Hercules, CA). Cytokines and chemokines in the lumbar spinal cord were quantified using the SearchLight Proteome Array (Aushon Biosystems, Billerica, MA). The Search Light Proteome Array is a multiplexed sandwich ELISA that was

customized to contain nine capture antibodies specific to IL-10, IL-12p70, IL-17, 1L-1α, 1L-1β, IL-2, MCP-1, TNF-α, IL-1β and TGF-β. In addition, TNF-α levels in the serum and sciatic nerve, gastrocnemius, and hindpaw plantar skin homogenates were quantified using a mouse TNF-α ELISA kit (eBioscience, San Diego, CA).

Immunohistochemistry for Microglial activation

Microglial activation in the lumbar spinal dorsal horn was assessed using an antibody to Iba-1 (Wako Chemicals, Richmond, VA). Mice were deeply anesthetized with Avertin (1.23% v/v tribromoethanol; 2.5% *tert*-amyl alcohol; 200µl/10 g body weight) and transcardially perfused with 4% paraformaldehyde. The spinal cord was dissected, post-fixed overnight in 4% paraformaldehyde, rinsed in PBS (pH 7.4), and cryoprotected in 30% sucrose. After freezing in OCT, spinal cords were sectioned on a cryostat at 20 µm and mounted on Superfrost Plus slides (Fisher Scientific, Pittsburgh, PA) and stored at -20 ^{0}C. After thawing for 5 minutes a room temperature, sections were covered with blocking solution (0.5% porcine gelatin, 1.5% normal donkey serum, and 0.5% Triton-X in Superblock buffer; Pierce) for one hour and then incubated overnight with Iba-1 primary antibody at 4^{0}C. All sections were rinsed with PBST and then incubated with donkey anti-rabbit fluorochrome-conjugated secondary antibody (Santa Cruz, Santa Cruz, CA) diluted in PBS and blocking solution for 1 hr at room temperature. Following wash in PBS, slides were rinsed in slides were rinsed in deionized distilled H_2O, coverslipped and stored at 4 ^{0}C. Optical density thresholds were manually selected for positively labeled tissue and immunoreactivity was quantified in the dorsal horn according to Popovich et al [247]. Image analysis was performed using Nikon Elements software and microglial activation was

reported here as the proportional area of tissue occupied by Iba-1 positive cells within the dorsal horn after the optical threshold was applied.

Statistics

Data were analyzed using a two-factor analysis of variance (ANOVA) or repeated measures ANOVA with Fisher's test of least square difference post-hoc comparisons. Statistical significance was set at $P < 0.05$.

4. Results and Figures

Body Weight and Glucose

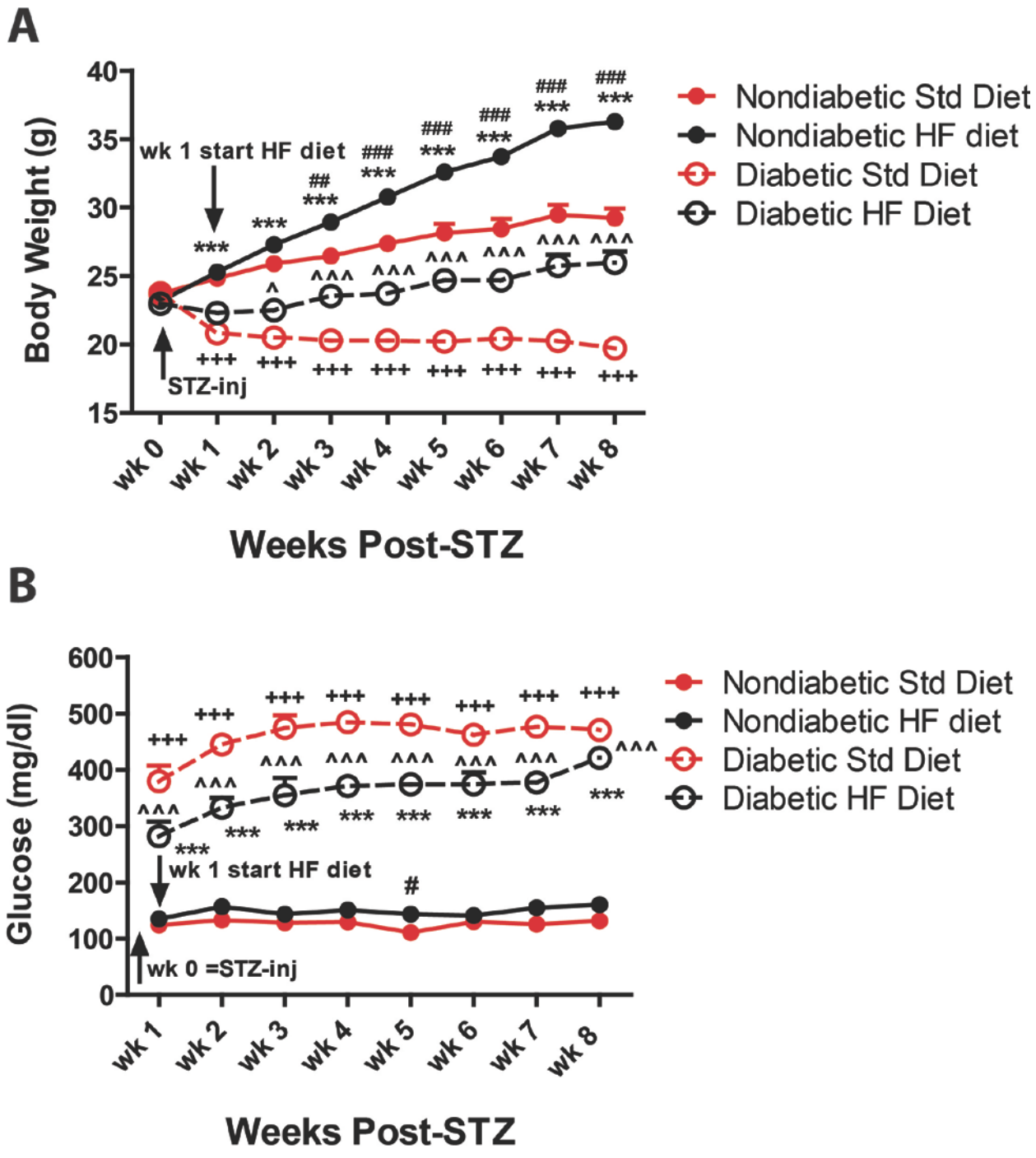
Body weight and glucose levels in this cohort of mice followed a nearly identical progression to the cohort of mice described in Chapter 2. Consistent with results reported in Chapter 2, both groups of diabetic mice weighed less compared to the nondiabetic mice on the equivalent diet (Figure 1A). Again, the diabetic high-fat-fed group gained weight while the diabetic mice fed the standard diet lost weight during the 8-week study (Figure 1A). In addition and similar to the cohort described in Chapter 2, nondiabetic mice fed the standard diet gained approximately 5g on average while nondiabetic high-fat-fed mice gained significantly more weight (approximately 13 g on average) over the course of the 8 week study (Figure 1A).

Typical of STZ-induced diabetic mice and consistent with the previous cohort, both groups of diabetic mice were severely hyperglycemic compared to their nondiabetic

Figure 1: The effects of STZ-induced diabetes and high-fat diet on body weight and fasting blood glucose.

Both diabetic groups display characteristic signs of diabetes including lower body weight and severe hyperglycemia compared to nondiabetic mice. High-fat fed mice gain more weight and have lower glucose levels than their standard diet counterparts. A) Body weight and B) Glucose. Data are presented as means ± SEM (n = 14 – 23 mice per group). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ for NdStd vs. NdHF, $^{+++}P < 0.001$ for NdStd vs. DbStd, ***$P < 0.001$: NdHF vs. DbHF, and ^$P < 0.05$, and ^^^$P < 0.001$ for DbStd vs. DbHF.

Figure 1


A
Body Weight (g)
40
35
30
25
20
15
wk 1 start HF diet
STZ-inj
wk 0
wk 1
wk 2
wk 3
wk 4
wk 5
wk 6
wk 7
wk 8
Weeks Post-STZ
Nondiabetic Std Diet
Nondiabetic HF diet
Diabetic Std Diet
Diabetic HF Diet
B
Glucose (mg/dl)
600
500
400
300
200
100
0
wk 1 start HF diet
wk 0 =STZ-inj
wk 1
wk 2
wk 3
wk 4
wk 5
wk 6
wk 7
wk 8
Weeks Post-STZ
Nondiabetic Std Diet
Nondiabetic HF diet
Diabetic Std Diet
Diabetic HF Diet

counterparts (Figure 1B). Despite severe hyperglycemia, diabetic mice fed the high-fat diet had significantly lower glucose levels than diabetic mice fed the standard diet (Figure 1B). In contrast, nondiabetic mice fed the high-fat diet had higher glucose levels than nondiabetic mice fed the standard diet throughout the duration of the study, but this difference was only significantly different at week 5 (Figure 1B). Although the glucose levels in the NdHF group were very similar in this cohort and the previous cohort, glucose levels were significantly higher in NdHF vs. NdStd at weeks 5, 7, and 8 in the previous cohort (Chapter 2, Figure 1B).

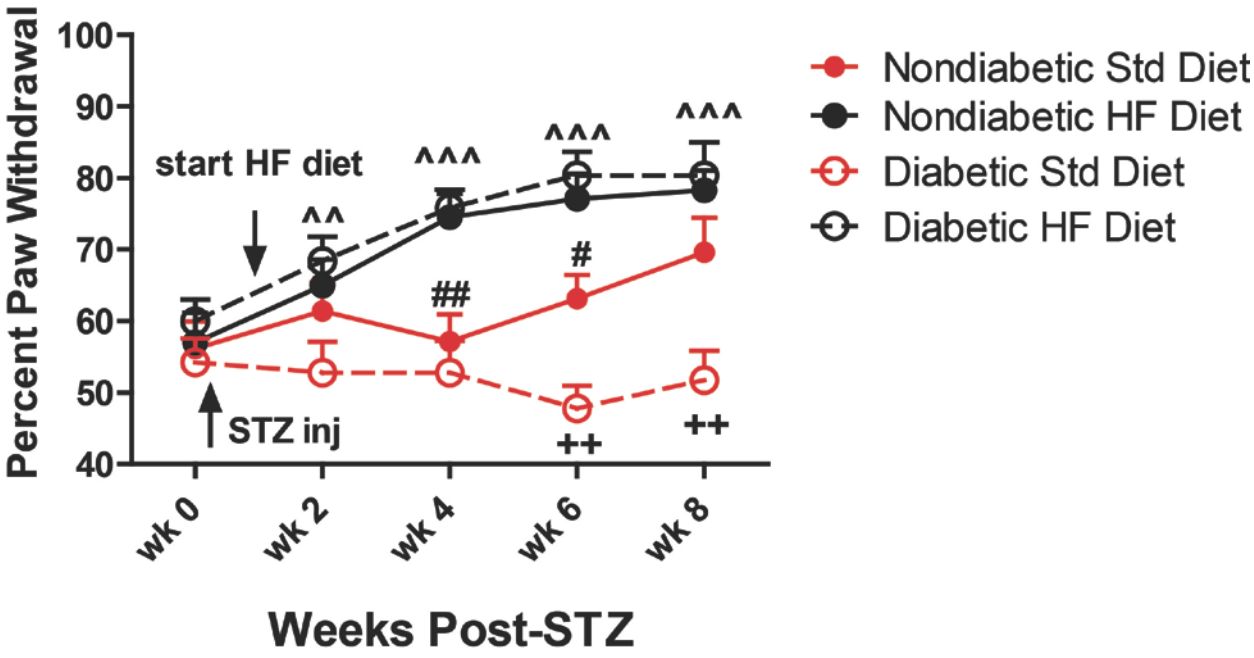
Nondiabetic and Diabetic Mice Fed a High-Fat Diet Develop Mechanical Hyperalgesia

Consistent with previous studies in this insensate model of diabetic neuropathy, STZ-induced diabetic C57Bl/6 mice lose mechanical sensation when consuming a standard low-fat diet (Figure 2) [181-184]. In stark contrast and congruent with results reported in Chapter 2, STZ-induced diabetic C57Bl/6 mice develop robust mechanical hyperalgesia when fed a high-fat diet (Figure 2) [30]. Nondiabetic mice fed the high-fat diet also developed robust mechanical hyperalgesia (Figure 2). Increased percent paw withdrawal reached statistical significance in DbHF at week 2 (compared to DbStd) and at week 4 in NdHF (compared to NdStd). Importantly, both NdHF and DbHF mice exhibited mechanical hyperalgesia and this behavioral phenotype was equivalent between these two groups (Figure 2). Thus, diabetes did not exacerbate high-fat diet induced mechanical hyperalgesia.

Figure 2: A High-fat diet induces mechanical hyperalgesia in nondiabetic and diabetic mice.

Behavioral responses to repeated applications of a mechanical stimulus (1.4 g von Frey monofilament). Data are presented as means ± SEM (n = 17 – 23 mice per group). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ for NdStd vs. NdHF, $^{++}P < 0.01$ for NdStd vs. DbStd, ^^P < 0.01 and ^^^$P <$ 0.001 for DbStd vs. DbHF.

Figure 2


Percent Paw Withdrawal
100
90
80
70
60
50
40
start HF diet
STZ inj
^^
^^^
^^^
^^^
##
#
++
++
wk 0
wk 2
wk 4
wk 6
wk 8
Weeks Post-STZ
Nondiabetic Std Diet
Nondiabetic HF Diet
Diabetic Std Diet
Diabetic HF Diet

High-Fat Diet Induced Mechanical Hyperalgesia is Not Accompanied by Spinal Inflammation

We predicted high-fat diet induced mechanical hyperalgesia would be accompanied by spinal inflammation and microglial activation thus contributing to central sensitization and nociceptive behavior. Sensory neurons that innervate the lower limbs and hind paws synapse in the dorsal horn of the spinal cord, thus we specifically examined microglial activation in the lumbar spinal dorsal horn and inflammatory mediators in the lumbar spinal cord. Results obtained from multiplex ELISA performed on lumbar spinal cord homogenates indicated no significant differences among groups in cytokines or chemokines: Il-1α, Il-1β, Il-2, TGF-β, Il-10, Il-12p70, Il-17, MCP-1, or TNF-α (Figure 3). Furthermore, there were no differences among groups in Iba-1 immunoreactivity in the lumbar spinal dorsal horn (Figure 4) suggesting that microglial activation does not contribute to diet-induced mechanical hyperalgesia in high-fat fed nondiabetic or diabetic mice.

TNF-α levels in Serum and Peripheral Tissues

Because TNF-α is often increased in inflammatory pain models [248, 249] and is typically elevated in the serum and periphery in obese humans and rodents [250-254] ,we assessed TNF-α in the serum and peripheral tissues. In contrast to our prediction, TNF-α was reduced in NdHF and DbStd groups compared to the NdStd group (Figure 5A). However, it should be noted that there were only 2 mice in the DbStd group for the serum measurement. The *n* was especially small for this group because serum volumes were too low in several samples due to use in previous experiments. Thus, results should be interpreted carefully. In addition,

Figure 3: Mechanical hyperalgesia is not accompanied by altered cytokine or chemokine expression in the lumbar spinal cord.

Cytokines and chemokines were measured by multiplex ELISA at 8 weeks post-STZ and after 8 weeks of the high-fat diet. Data are presented as means ± SEM (n = 7-10 mice per group). No significant differences among groups.

Figure 3

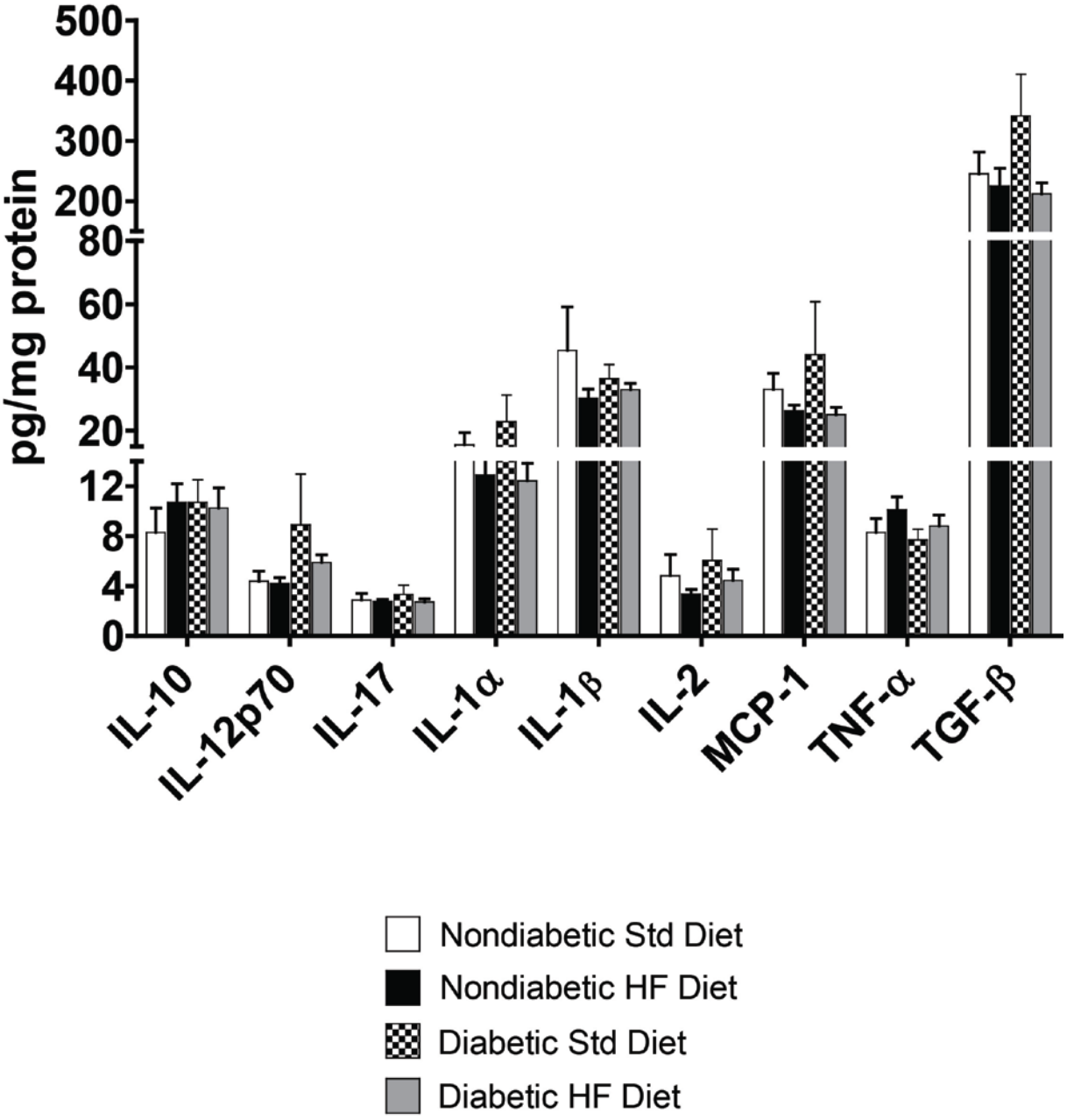
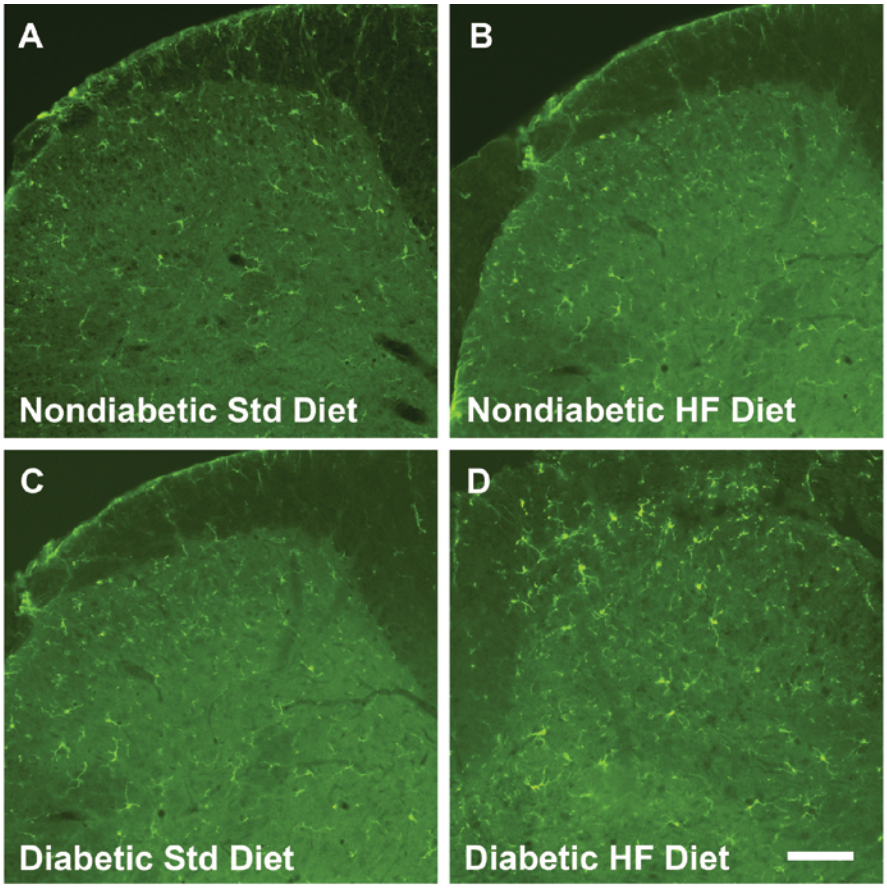

pg/mg protein
500
400
300
200
80
60
40
20
12
8
4
0
IL-10
IL-12p70
IL-17
IL-1α
IL-1β
IL-2
MCP-1
TNF-α
TGF-β
Nondiabetic Std Diet
Nondiabetic HF Diet
Diabetic Std Diet
Diabetic HF Diet

Figure 4: Microglial activation in the lumbar spinal dorsal horn is not impacted by diabetes or a high-fat diet.

Microglial activation was quantified using immunohistochemistry for Iba-1 in the lumbar dorsal horn of the spinal cord. Tissues were harvested at 8 weeks post-STZ and after 8 weeks of high-fat feeding. A-D) Representative images showing sections of the lumbar dorsal horn used to quantify microglial activation. A) Nondiabetic standard diet. B) Nondiabetic high-fat diet. C) Diabetic standard diet. D) Diabetic high-fat diet. Scale bar = 100μm. E) Quantification of Iba-1 immunoreactivity within the lumbar dorsal horn. Data are presented as means ± SEM. (n = 5-7 mice per group). No significant differences among groups.

Figure 4


A
Nondiabetic Std Diet
B
Nondiabetic HF Diet
C
Diabetic Std Diet
D
Diabetic HF Diet

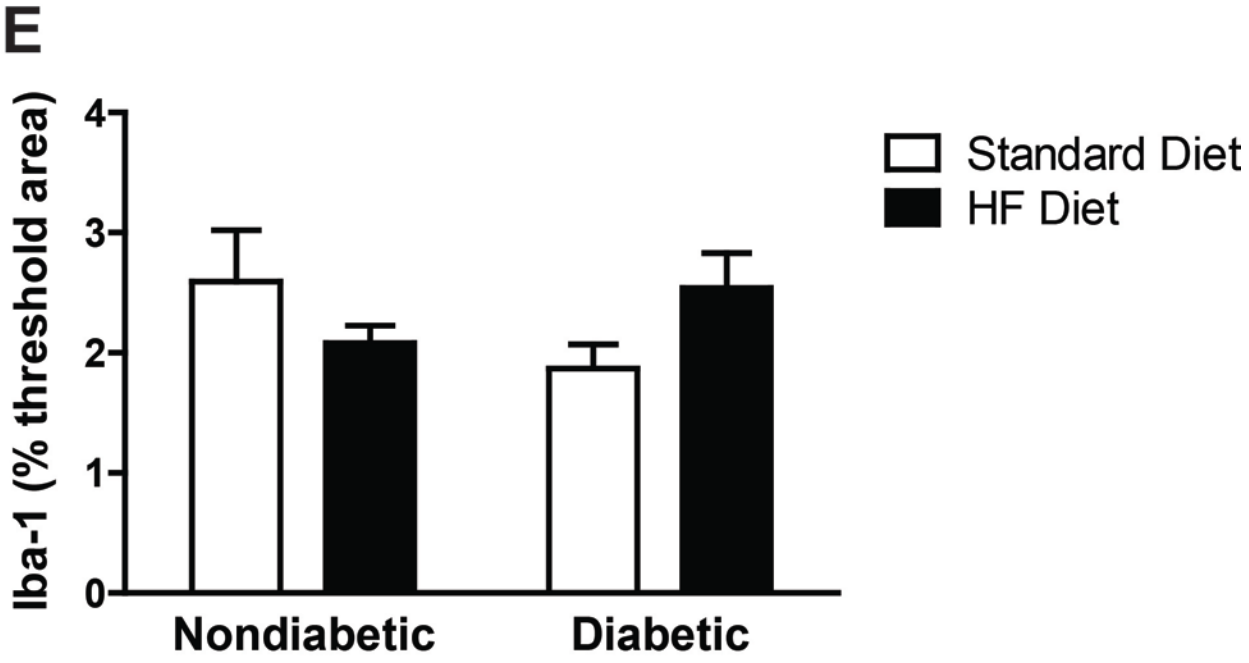
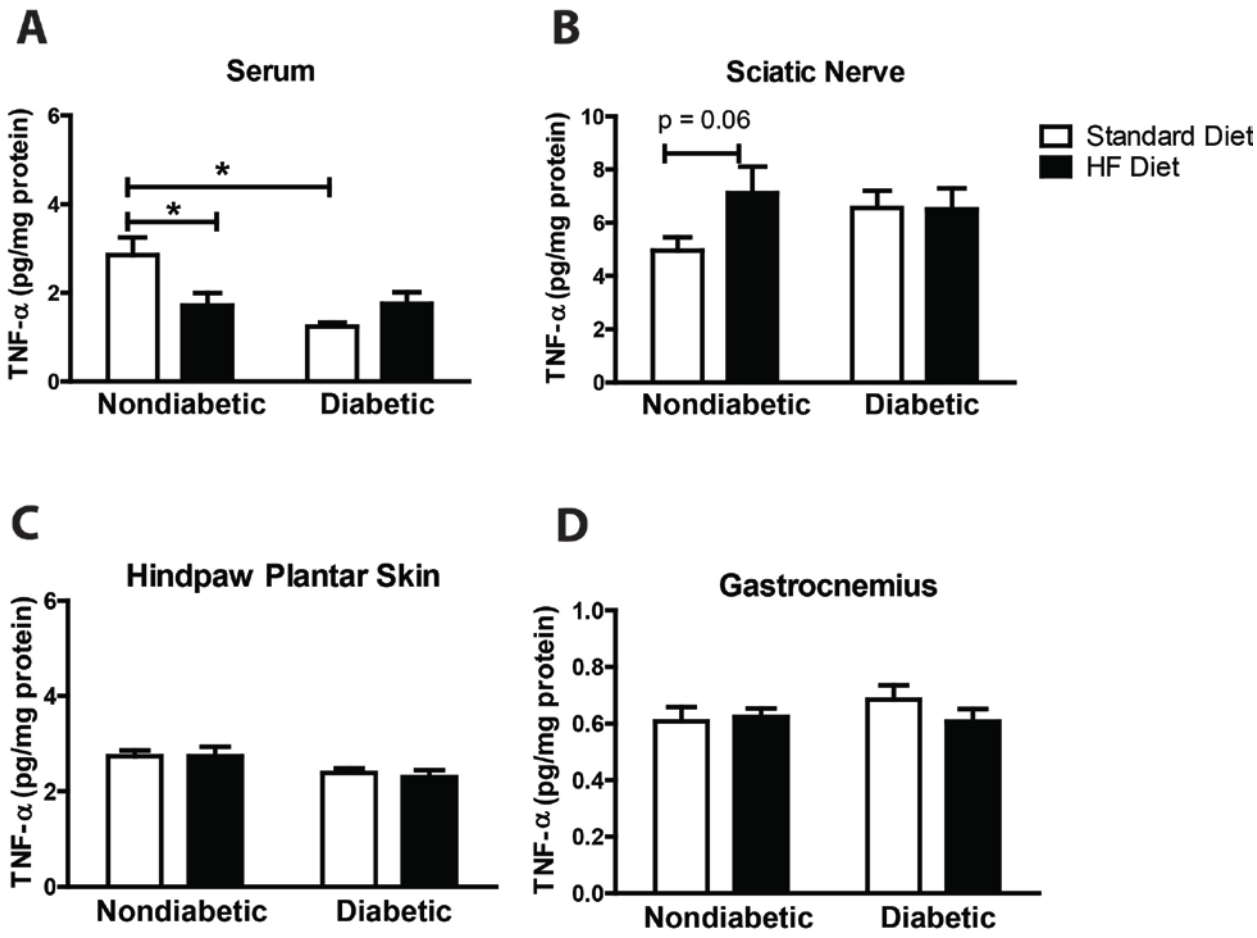

E
Iba-1 (% threshold area)
0
1
2
3
4
Nondiabetic
Diabetic
Standard Diet
HF Diet

Figure 5: TNF-α expression in serum and peripheral tissues.

TNF-α levels were measured by ELISA at 8 weeks post-STZ and after 8 weeks of high-fat feeding. A) Serum (*n* = 2-8 mice per group). B) Sciatic nerve (*n* = 10-13 mice per group). C) Hindpaw plantar skin (*n* = 3-9 mice per group). D) Gastrocnemius (*n* = 10-13 mice per group). Data are presented as means ± SEM. *P < 0.05.

Figure 5


A
Serum
TNF-α (pg/mg protein)
6
4
2
0
*
*
Nondiabetic
Diabetic
B
Sciatic Nerve
p = 0.06
TNF-α (pg/mg protein)
10
8
6
4
2
0
Nondiabetic
Diabetic
Standard Diet
HF Diet
C
Hindpaw Plantar Skin
TNF-α (pg/mg protein)
6
4
2
0
Nondiabetic
Diabetic
D
Gastrocnemius
TNF-α (pg/mg protein)
1.0
0.8
0.6
0.4
0.2
0.0
Nondiabetic
Diabetic

there were no significant differences among groups in TNF-α levels within sciatic nerve (Figure 5B), the hindpaw plantar skin (Figure 5C).

5. Discussion

Inflammation is considered to be a major factor underlying the pathogenesis of type 2 diabetes, and has also been proposed as a major factor in the development of diabetic neuropathy in animal models [102, 255]. In addition, human patients with diabetic neuropathy have increased serum cytokine levels compared to healthy control subjects [93, 256]. Furthermore, serum pro-inflammatory cytokine levels are higher in patients with painful neuropathy compared to patients with painless neuropathy [93, 95, 256]. Purwata et al. also specifically reported higher serum TNF-α levels in patients with severe pain versus mild pain [93]. Consistent with these clinical findings, TNF-α levels in the lumbar dorsal spinal cord of diabetic rats were negatively correlated with paw withdrawal threshold suggesting elevated TNF-α levels may contribute to mechanical hypersensitivity [257].

High-fat-fed nondiabetic rodents display signs of diabetic neuropathy including nerve conduction velocity deficits, altered sensorimotor behavior, and reduced intraepidermal nerve fiber density [5, 23, 30, 258, 259]. High-fat-fed mice exhibit mechanical hyperalgesia, indicative of increased nociception, characteristic of a painful phenotype of diabetic neuropathy. Although high-fat diet induced mechanical hyperalgesia has been documented in the literature [23, 30], the mechanism underlying this diet-induced neuropathy phenotype is unknown. The aim of this study was to investigate the role of inflammation as a potential mechanism underlying high-fat diet induced mechanical hyperalgesia. Here, we report high-fat diet induced mechanical

hyperalgesia is not accompanied by spinal inflammation or microglial activation in nondiabetic or diabetic mice after 8 weeks of diabetes and/or high-fat feeding.

High-Fat Diet Induced Mechanical Hyperalgesia is Not Accompanied by Inflammation

Contrary to our hypothesis, we did not observe changes in chemokines or cytokines in the lumbar spinal cord of diabetic or high-fat-fed mice. These results were surprising and are in contrast to the large body of work documenting the association of a pro-inflammatory cytokine profile with painful diabetic neuropathy (reviewed in [88]) and reports of high-fat diet induced inflammation. However, only one of the studies reviewed by Wilson and colleagues [88] examined cytokine levels in the spinal cord and this study was done in rats after 4 weeks of diabetes [257]. It is plausible that our results were different because cytokines were measured later in the course of diabetes (8 weeks post-STZ) and were assessed in mice rather than rats.

In the current study, we also examined TNF-α levels in serum and several peripheral tissues because TNF-α is a pro-inflammatory cytokine that is commonly implicated in inflammatory pain [248, 249], diabetic neuropathy [89, 93, 99, 256] , and obesity induced inflammation [250-254]. In contrast with reports documenting increased TNF-α levels in painful neuropathy in human patients and STZ-induced diabetic rodents [89, 93, 99, 256] TNF-α levels in the serum, sciatic nerve, gastrocnemius, or hindpaw plantar skin were not significantly increased in NdHF, DbStd, or DbHF. In fact, serum TNF-α levels were reduced in NdHF and DbStd compared to nondiabetic mice fed the standard diet. It should be noted that serum from only 2 mice in the DbStd group were assessed for TNF-α, thus changes in serum TNF-α in the DbStd group should be interpreted with caution. Interestingly, in the sciatic nerve,

the P-value was 0.06 for NdStd vs. NdHF, indicating a trend toward increased TNF-α in the sciatic nerve of nondiabetic mice that consumed a high-fat diet. However, there was not a trend toward increased sciatic TNF-α in DbHF, the other group that exhibited mechanical hyperalgesia, suggesting that elevated sciatic TNF-α is not driving the painful phenotype in both nondiabetic and diabetic mice.

In the current study, STZ-diabetic mice fed the standard diet study exhibited insensate neuropathy, which is consistent with previous studies in this mouse strain [181-184]. Many of the reports of increased TNF-α levels are in STZ-diabetic rodents that display a painful neuropathy phenotype. Therefore, we expected to see inflammatory changes in the high-fat-fed nondiabetic and diabetic mice in conjunction with mechanical hyperalgesia and did not expect to see inflammatory changes the diabetic mice fed the standard diet. However, in support of our results, Saleh and colleagues recently reported decreased TNF-α levels in DRG from STZ-diabetic rats and posited that reduced TNF-α expression in the DRG may contribute to impaired nerve regeneration and collateral sprouting in type 1 diabetes [108]. Again, differences in our results compared to previous reports may be attributed to different time points at which experiments were performed.

It is noteworthy to mention that none of the previous studies have examined cytokine levels in STZ-induced diabetic mice fed a high-fat diet, which we report here. Analysis of additional cytokines and chemokines in the serum and peripheral tissues would better characterize the inflammatory or non-inflammatory state. Since adipose inflammation has been implicated as a major contributor to inflammation associated with obesity or a high-fat diet [78, 124, 260-263], future studies will specifically assess inflammatory factors in adipose tissue. Furthermore, the DRG should be included in future inflammatory profiling because the DRG

could be a key site where inflammation might induce mechanical hyperalgesia in the hindpaws. Moreover, studies designed to assess central and peripheral nervous system inflammation at the onset of hyperalgesia may reveal early inflammatory changes that are ameliorated by compensatory mechanisms after several weeks.

High-Fat Diet Induced Mechanical Hyperalgesia is Not Accompanied by Microglial Activation

In models of inflammatory pain, microglial activation often is viewed to go hand in hand with spinal and peripheral inflammation [239, 264-267]. Microglia can be a source of pro-inflammatory cytokines and have been deemed necessary to produce pathological pain in laboratory animals [243-245]. Spinal glial activation has been reported in rodent models of diabetic neuropathy that display mechanical allodynia but these studies are few and only one study evaluated mice [246, 268, 269]. In the current study, there were no differences in microglial activation among groups, indicating that diabetes nor the high fat diet affected microglial activation in the lumbar spinal dorsal horn. In support of this, Gardiner and colleagues have recently reported that microglial activation is not increased in the lumbar dorsal spinal horn of STZ-induced diabetic C57Bl/6 mice (unpublished data). In addition, there is some controversy surrounding measurement of microglial activation. Activated microglia can hypertrophy, increase expression of activation markers, undergo morphological changes, an/or increase in number [248, 270, 271]. However, microglial activation has also been described as increased microglial number with retraction of processes [272]. In fact, Jonas and colleagues recently reported that microglial undergo several morphological stages of activation, and were the first to describe these stages in detail [272]. Thus, the method used to quantify Iba-1 immunoreactivity should be chosen carefully. We chose to use percent threshold area to

quantify microglial activation because this method is commonly used in injury models of neuropathic pain [247, 248]. Although Gardiner and colleagues used a slightly different method and counted microglia on a grid, our results were still similar suggesting that microglial activation is not increased in STZ-induced diabetic mice fed a high-fat diet. To our knowledge, ours is the first study to examine microglial activation in high-fat-fed nondiabetic or diabetic mice.

In conclusion, a high-fat diet induced mechanical hyperalgesia was not accompanied by increased spinal inflammation, microglial activation, or increased TNF-α levels in the periphery suggesting inflammation is not driving mechanical hyperalgesia in this model. Importantly, this was the first time that cytokines, chemokines, and microglial activation have been assessed in this novel combination STZ-induced diabetes/high-fat diet model of diabetic neuropathy. Although there were not significant inflammatory changes at this time point, it is plausible to suggest that inflammation occurred earlier in the study near the initiation of behavioral changes and by the time we examined tissues at week 8, these changes were already downregulated by compensatory mechanisms. Future studies will investigate inflammatory mediators in additional tissues including the DRG and adipose at the initiation of behavioral changes in nociception.

CHAPTER 5

The Effects of Exercise on Insulin Signaling in Mice Fed a High-Fat Diet

1. Abstract

Insulin resistance has been thoroughly characterized in insulin-dependent tissues including muscle, fat, and liver in diabetic animals and humans. Emerging evidence demonstrates that insulin resistance occurs in neurons and neuronal insulin resistance has been proposed as a novel etiological factor that contributes to neurodegeneration and dysfunction that occurs in diabetic neuropathy. The purpose of this study was to determine if high-fat-fed nondiabetic mice that display signs of painful neuropathy also exhibit neuronal insulin resistance. In addition, we tested the effects of exercise on insulin signaling and mechanical sensitivity in nondiabetic mice that were fed a high-fat diet. Adult C57Bl/6 mice consumed a standard or high-fat diet for four weeks. In addition, half of the high-fat-fed nondiabetic mice had ad libitum access to a running wheel for the entire duration of the four-week study. After stimulation via intra-peritoneal insulin injection, insulin signaling in the lumbar DRG, sciatic nerve, and gastrocnemius was evaluated using Western blots to quantify Akt activation. Although mechanical sensitivity was not significantly different among groups over the course of the four-week study in this cohort of animals, we have previously observed mechanical hyperalgesia in this mouse strain and these behavioral changes begin to appear after 4 weeks of high-fat feeding. Exercise prevented increased body weight, glucose and insulin levels, and HOMA-IR in nondiabetic mice fed a high-fat diet. Akt activation in the DRG and sciatic nerve was not significantly affected by the high-fat diet or exercise. In contrast, Akt activation was significantly blunted in the gastrocnemius of high-fat-fed sedentary mice and this impairment was partially restored by exercise. In conclusion, exercise is an effective intervention to combat the deleterious effects of a high-fat diet on insulin resistance and other metabolic variables.

Together, these data demonstrate the potent ability of exercise to ameliorate or diminish metabolic derangements induced by a high-fat diet.

2. Introduction

Insulin resistance is a hallmark feature of type 2 diabetes and has recently been reported to occur in type 1 diabetes [110-113]. Insulin resistance in the setting of diabetes has been well characterized in insulin-dependent tissues including muscle, fat, and liver [114-116], but has not been thoroughly studied in the peripheral nervous system. Neurons respond to insulin, but neuronal glucose uptake occurs in an insulin-independent manner that is different than glucose uptake in muscle, fat and liver [117, 118]. Although neurons do not require insulin for glucose uptake, insulin is an important neurotrophic factor that neurons rely on for growth [121, 122], regeneration [123-125], and maintenance of mitochondrial function [21, 66]. Blunted insulin signaling and diminished insulin-stimulated neurite outgrowth has been reported in the DRG from diabetic rodents [117, 132]. Therefore, recently, impaired neuronal insulin signaling has been proposed as an etiological factor involved in neuronal damage and neurodegeneration that occurs in diabetic neuropathy [109, 132].

Insulin resistance in peripheral tissues is consequence of a high-fat diet in rodents and humans [273-278]. There are also several studies reporting the beneficial effects of exercise on insulin sensitivity in insulin-dependent tissues in diabetic patients and high-fat-fed animals . [150, 155, 156]. However, to our knowledge, no one has investigated the effects of a high-fat diet or exercise on insulin signaling in the peripheral nervous system. We have previously discovered that a high-fat diet induces signs of painful neuropathy in sedentary mice [279]. Thus, further study on peripheral nervous system insulin signaling in high-fat models of diabetic neuropathy is warranted. Therefore, the aim of this study was to determine whether blunted peripheral nervous system insulin signaling occurs in conjunction with the behavioral changes

that occur in this high-fat model of diabetic neuropathy. In addition, we investigated the effects of exercise on sensorimotor behavior and insulin signaling in high-fat-fed mice.

3. Experimental Procedures

Animals, Diet, and Voluntary Exercise

Seven week-old male C57Bl/6 mice were purchased from Charles River (Wilmington, MA). Mice were housed two mice per cage under pathogen free conditions, and placed on a 12:12h light/dark cycle in the research support facility at the University of Kansas Medical Center. All animals had ad libitum access to food and water and were fed a standard diet (8604; Harlan Teklad, Madison Wisconsin; 14% kcals from fat, 32% protein, and 54% carbohydrate) or high-fat diet (07011; Harlan Teklad; 54% kcals from fat comprised of lard and corn oil, 21% protein, and 24% carbohydrate) for 4 weeks. Half of animals on the high-fat diet were randomly assigned to the exercise group. Exercised mice were housed individually in a cage with ad libitum access to a running wheel for the duration of the 4-week study. Mice in both the sedentary and exercise groups began the high-fat diet (and the exercise group was placed in the exercise wheel cages) immediately following baseline behavioral testing. Each wheel revolution was recorded in thirty-minute intervals with the Vital View Data Acquisition System (Mini Mitter, Bend, OR) throughout the duration of the study. All protocols and procedures were approved by the University of Kansas Medical Center Animal Use and Care Committee. Treatment groups are abbreviated throughout as follows: standard diet sedentary (SS); high-fat diet sedentary (HS); high-fat exercised (HE).

Behavioral Testing for Mechanical Sensitivity

Behavioral testing was performed as described in the previous chapters. In this specific study, behavioral testing was performed at baseline and every week thereafter for four weeks. Mice were placed on an elevated wire mesh screen (55 cm above table), enclosed individually in clear plastic cages, and mechanical sensitivity was assessed using a 1.4 g Semmes Weinstein von Frey monofilament (Stoelting, Wood Dale, IL) that was applied 6 times to each hind paw footpad. A mean percent withdrawal from a total of 12 applications was calculated per mouse and used to calculate group means.

Glucose, Insulin, and HOMA-IR

Following a three hour fast, blood was collected at baseline and at the end of the 4-week study immediately before insulin stimulation. Samples were assayed for glucose using glucose diagnostic reagents (Sigma, St. Louis, MO). For insulin measurements, blood was collected into a heparinized microcapillary tube, then pipetted into an Eppendorf tube, and centrifuged for 15 minutes at 3,000 x *g*. Plasma was removed and frozen at -80 ^{o}C. Insulin levels were measured using ELISA (mouse insulin Elisa, Alpco, Salem, NH). Fasting insulin and fasting glucose levels were used to calculate the homeostatic model assessment of insulin resistance (HOMA-IR), a measure of insulin resistance. The following equation was used to calculate HOMA-IR values: Blood (Glucose (mg/dl) X (Serum Insulin (uU/mL)/405 [280].

Insulin Stimulation

Following a three hour fast, sterile PBS (vehicle) or 10 U/kg Humulin R insulin was administered to mice from the SS, HS, and HE groups. Humulin R insulin was diluted to 0.001

U/μL in sterile PBS and 10 μl/g was injected via a single intra-peritoneal injection. Thirty minutes after insulin injection, mice were anesthetized with isofluorane and decapitated. Blood was collected from the decapitation pool for post-insulin stimulation glucose quantification.

Western Blots for Akt Activation

After the 30 minute insulin stimulation period, mice were killed as described above and the DRG, sciatic nerve, and gastrocnemius were harvested, snap frozen in liquid nitrogen, and stored at -80°C. Tissues were lysed with cell lysis buffer (137 mM NaCl, 20 mM Tris [pH 8.0], 1% NP40, and 10% glycerol) containing protease inhibitors (0.5 mm sodium vanadate, 1 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF) and protein was extracted while tissue lysates were kept on ice for 1 hour and vortexed every 10 minutes. Samples were then centrifuged at 10,000 *g* for 10 minutes at 4^0 C and the protein concentration of the supernatant was measured with a Bradford assay (Bio-Rad, Hercules, CA).

Samples were prepared to achieve equivalent protein concentrations then boiled at 95-100 ^{0}C with Lane Marker Reducing Sample Buffer (Thermo Scientific, Waltham, MA) for 3 minutes. Samples containing 30 μg of protein were separated by electrophoresis (35 mA/gel, 0.75 hr, 4^0 C) on 4-15% gradient tris-glycine polyacrylamide gels (Bio-Rad) and transferred onto nitrocellulose membrane (400 mA, 1.5 hr, 4^0C). Nitrocellulose membranes were blocked for 1 hour at room temperature in blocking solution (5% non-fat powdered milk and 0.05% Tween-20 in 0.1 M phosphate buffered saline [PBST, pH 7.4]), followed by overnight incubation (4^0C) in primary antibody diluted in blocking solution (1% non-fat powdered milk and 0.05% 0.1 M PBS (pH 7.4)**.**

Membranes were probed using following antibodies: total Akt (Cell Signaling), phospho(Ser473)Akt (Cell Signaling), β-actin (Millipore). Membranes were washed in PBST, and then incubated in either anti-mouse or anti-rabbit horse radish peroxidase conjugate (Santa Cruz) for 1 hour at room temperature. Bands were visualized by enhanced chemiluminescence (ECL) using Supersignal West (Femto or Pico) Substrate (Pierce, Rockford, IL) and detected on X-ray film. Bands were quantified via densitometry using NIH Image J software.

Statistics

Data were analyzed using a two-factor analysis of variance (ANOVA) or repeated measures ANOVA with Fisher's test of least square difference post-hoc comparisons. Statistical significance was set at $P < 0.05$.

4. Results and Figures

Body Weight, Glucose, Insulin

Sedentary mice fed the high-fat diet had increased body weight (Figure 1A), glucose and insulin levels (Figure 2A-B), and HOMA-IR (Figure 2C) compared to sedentary mice on the standard diet. Although elevated glucose and hyperinsulinemia in high-fat sedentary mice suggested these mice were in a pre-diabetic state, glucose levels were not high enough to be classified as overt diabetes (Figure 2A-B). Body weight (Figure 1), glucose, insulin, and HOMA-IR (Figure 2A-C) were not different from standard diet sedentary mice in the high-fat exercise group, thus voluntary running wheel exercise prevented deleterious changes in all of these metabolic variables.

Figure 1: Exercise prevents excess weight gain in mice fed a high-fat diet.

Body weight was significantly higher in sedentary mice after 4 weeks of high-fat feeding compared to sedentary standard diet mice and high-fat exercised mice. Data are presented as means ± SEM (n = 19 – 21 mice per group). $^{###}P < 0.001$ for SS vs. HS and $***P < 0.001$ for HS vs. HE.

Figure 1

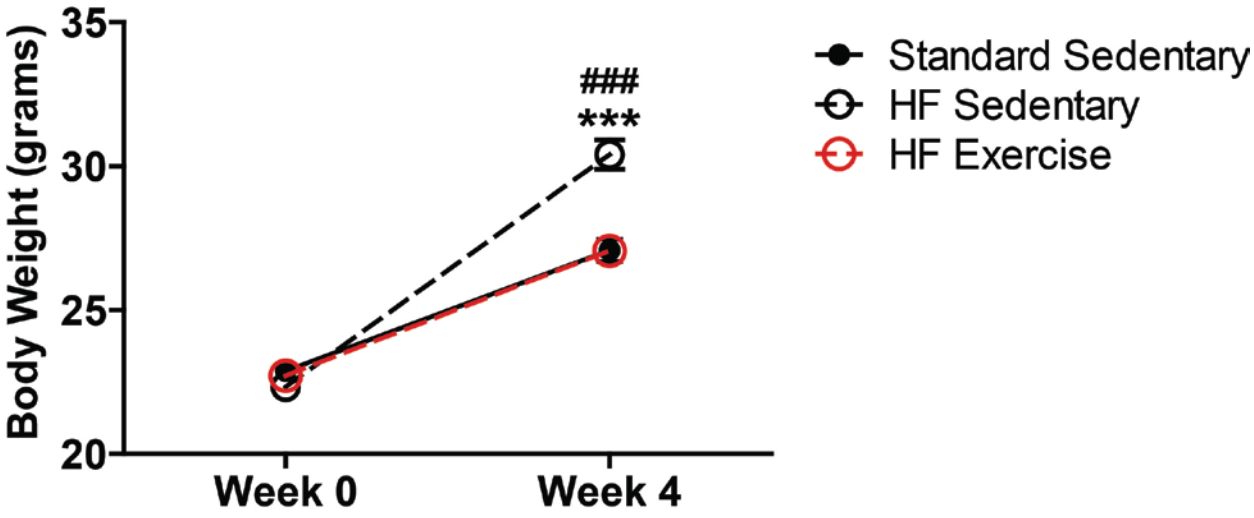
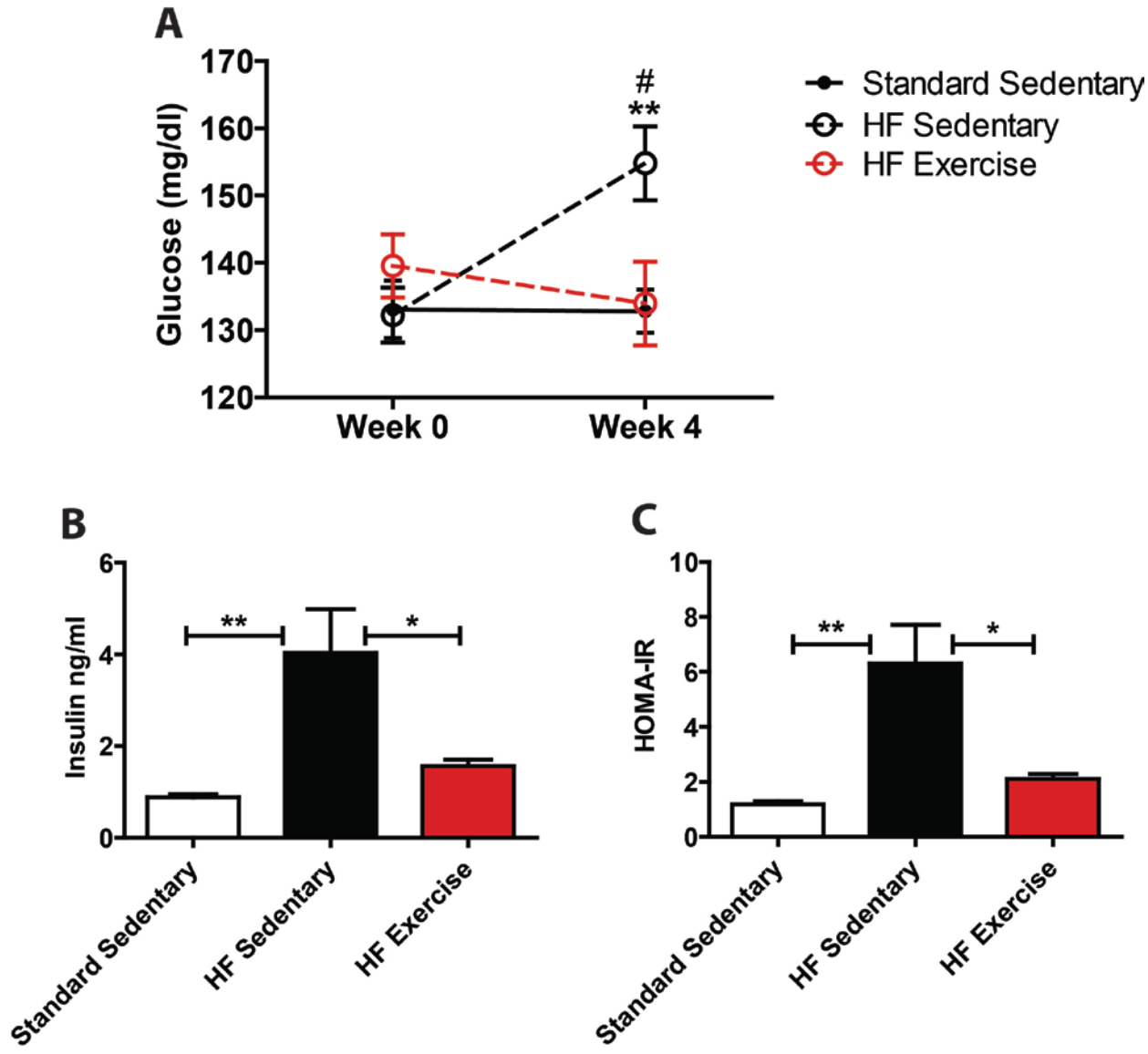

Body Weight (grams)
35
30
25
20
Week 0
Week 4
###
***
Standard Sedentary
HF Sedentary
HF Exercise

Figure 2: Exercise prevents metabolic defects in high-fat fed mice.

A) Blood glucose following a 3-hour fast. $^{\#}P < 0.05$ for SS vs. HS and $^{**}P < 0.01$ for HS vs. HE. B) Serum insulin after 3-hour fast. Blood was collected after 4 weeks of high-fat feeding and/or exercise. $^{*}P < 0.05$. C) Homeostatic model of insulin resistance (a measure of insulin resistance) after 4 weeks of high-fat feeding and/or exercise. $^{*}P < 0.05$. Data are presented as means ± SEM (n = 18-20 mice per group).

Figure 2


A
Glucose (mg/dl)
170
160
150
140
130
120
Week 0
Week 4
#
**
Standard Sedentary
HF Sedentary
HF Exercise
B
Insulin ng/ml
6
4
2
0
**
*
Standard Sedentary
HF Sedentary
HF Exercise
C
HOMA-IR
10
8
6
4
2
0
**
*
Standard Sedentary
HF Sedentary
HF Exercise

Exercise Distance and Timing

High-fat-fed mice had ad libitum access to a running wheel in their individual cages 24 hours per day, 7 days per week. However, due to their nocturnal nature, mice perform the bulk of their running during the dark portion of the 12 hr light/dark cycle that occurs from 6 pm to 6 am (Figure 3A). Daily running distance is calculated by multiplying the number of wheel turns by the diameter of the wheel and according to these calculations, on average, these mice ran 9.9 km per 24 hour day (Figure 3B). As illustrated in Figure 3B, running distance is similar to a bell curve over the course of the four weeks, with running distance increasing to peak at week 2 and tapering off by week 4.

Mechanical Sensitivity

Behavioral responses to twelve repeated applications of a 1.4 g von Frey monofilament were calculated as percent paw withdrawal. Although there were no significant differences among groups in this cohort of animals (Figure 4), sedentary high-fat-fed mice develop mechanical hyperalgesia compared to standard diet sedentary mice [30] and we have documented this in numerous cohorts in our laboratory. However, mechanical hyperalgesia typically first appears after 4 weeks of high-fat feeding and is more well established after 8 weeks of a high-fat diet. In some cohorts, increased mechanical sensitivity is statistically

significant at week 4 while in other cohorts, mechanical sensitivity is not different from standard diet mice. This inconsistency is due to the fact that this time point is near the initiation of behavioral changes, on the cusp of when mechanical hypersensitivity develops. Because we wanted to characterize insulin sensitivity at the initiation of behavioral changes, we specifically

Figure 3: Mice consistently engage in voluntary exercise throughout the duration of the 4-week study and perform the majority of their running between the hours of 6 pm and 6 am.

A) Twenty four hour exercise timeline in exercised mice fed a high-fat diet. B) Daily running distance in exercised mice fed a high-fat diet high. Data are presented as means ± SEM (n = 19). **$P < 0.01$ for week 2 vs. week 3. ***$P < 0.001$ for week 1 vs. week 2.

Figure 3

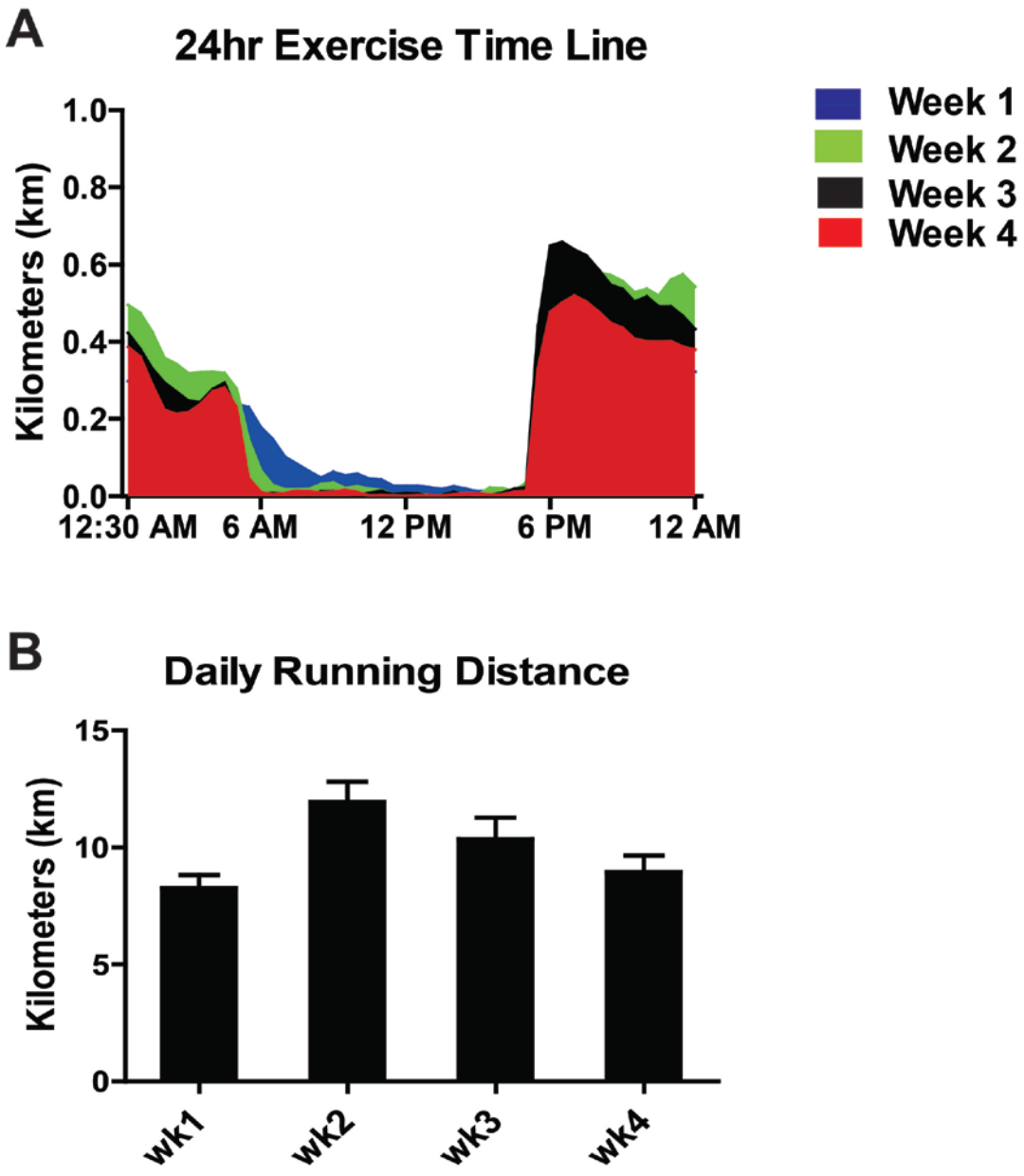
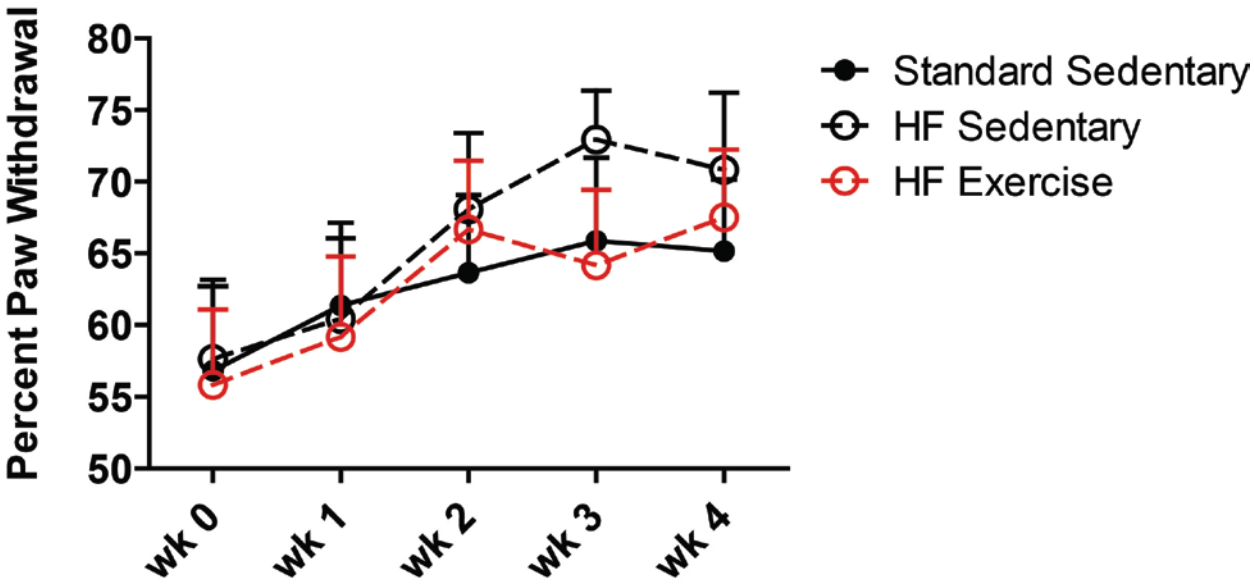

A
24hr Exercise Time Line
Kilometers (km)
1.0
0.8
0.6
0.4
0.2
0.0
12:30 AM
6 AM
12 PM
6 PM
12 AM
Week 1
Week 2
Week 3
Week 4
B
Daily Running Distance
Kilometers (km)
15
10
5
0
wk1
wk2
wk3
wk4

Figure 4: The effects of a high-fat diet on mechanical sensitivity in sedentary and exercised mice.

Mechanical sensitivity was assessed after 4 weeks of a high-fat diet and sedentary activity or exercise using 12 repeated applications of a 1.4 g von Frey monofilament. Data presented as means ± SEM (n = 10 – 12 mice per group). No significant differences.

Figure 4


Percent Paw Withdrawal
80
75
70
65
60
55
50
wk 0
wk 1
wk 2
wk 3
wk 4
Standard Sedentary
HF Sedentary
HF Exercise

chose to use 4 weeks of high-fat feeding. Exercise did not significantly alter mechanical sensitivity in high-fat-fed mice at these early time points in this short-term study (Figure 4).

Response to Insulin

In order to ensure the efficacy of the 30-minute insulin stimulation, we measured blood glucose levels before and after 30-minute insulin stimulation. Although there were no statistically significant differences among groups, glucose was reduced by 53% in high-fat-fed exercised mice compared to a 39% and 41% reduction in glucose after insulin stimulation compared to sedentary diet sedentary and high-fat-fed sedentary mice (Figure 5). This data points to increased insulin sensitivity in exercised mice compared to sedentary mice.

Akt Activation

Insulin signaling was evaluated after insulin stimulation by quantifying Akt activation (pAkt/total Akt) in peripheral tissues. In SS, HS, and HE, insulin injection elicited a significant increase in Akt activation in the DRG, sciatic nerve, and gastrocnemius compared to PBS injection in the corresponding group (Figure 6A-C). There were no significant differences among groups in pAkt/total Akt in the DRG (Figure 6A) or sciatic nerve (Figure 6B), indicating that the neither high-fat diet, nor exercise impacted insulin sensitivity in these tissues. In stark contrast, insulin stimulated Akt activation was significantly blunted in the gastrocnemius of high-fat-fed sedentary mice compared to standard diet sedentary mice (Figure 6C). Akt activation was significantly higher in the high-fat exercised mice compared to high-fat-fed sedentary mice, thus

Figure 5: The effects of insulin stimulation on glucose levels in sedentary and exercised mice fed a high-fat diet.

Following a 3 hour fast, mice were administered a single intraperitoneal injection of sterile PBS or insulin (0.1 U/kg) 30 minutes before sacrifice. Blood was collected immediately before injection and then immediately after the 30 minute stimulation period upon sacrifice. Response to injection was reported as percent change in glucose level and calculated according to the following formula: ([post-injection glucose level – pre-injection glucose level]

/ pre-injection glucose level) * 100. Data are presented as means ± SEM (*n* = 7-11 mice per each of 6 groups). No significant differences.

Figure 5

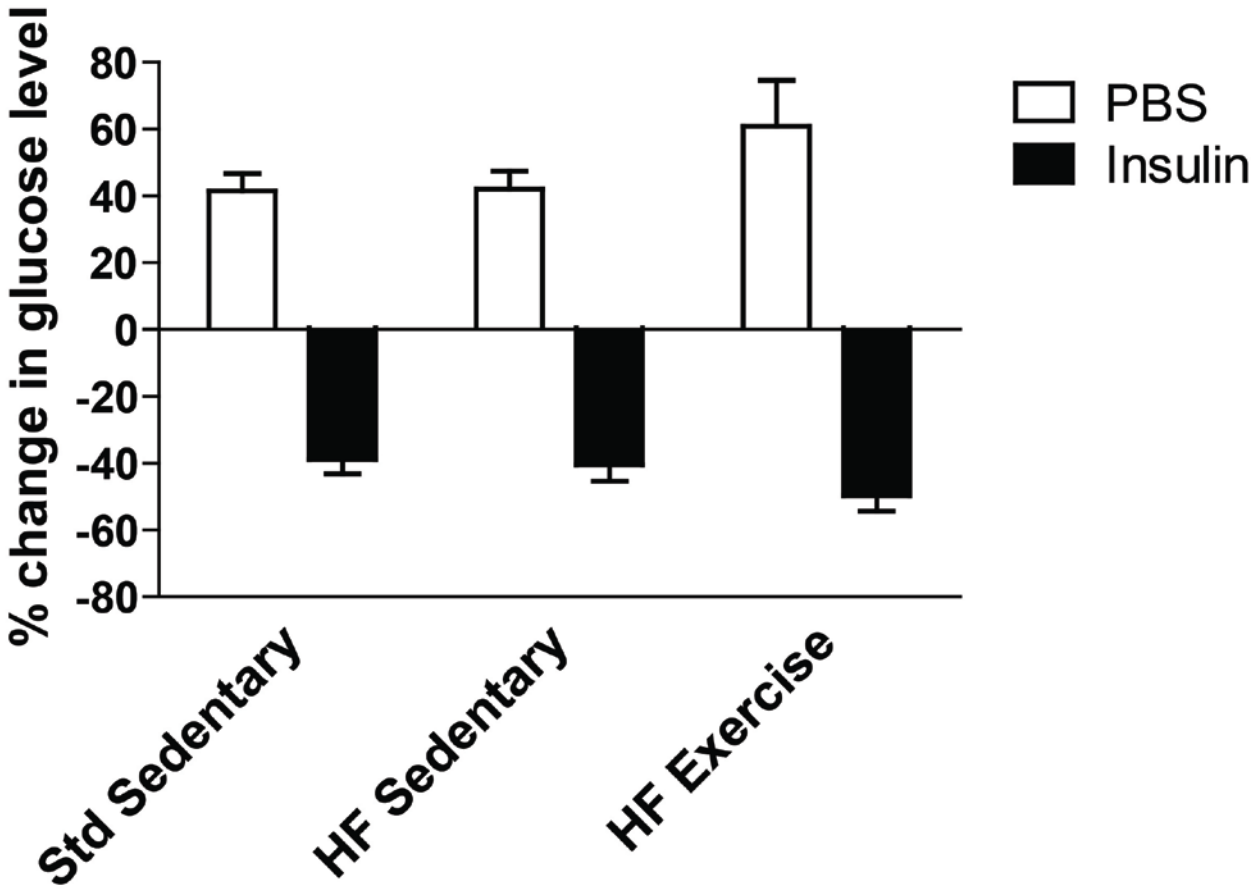
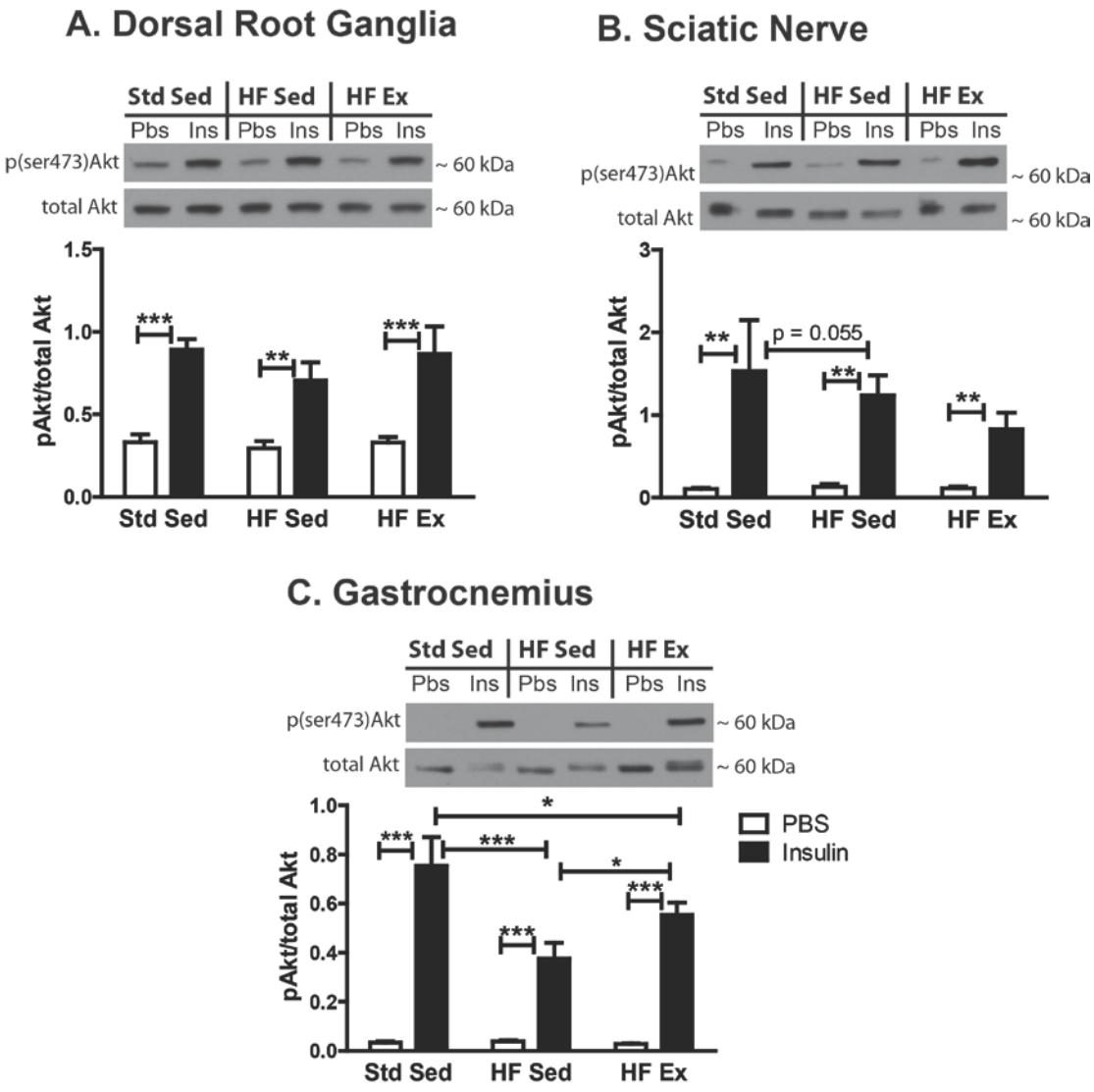

% change in glucose level
80
60
40
20
0
-20
-40
-60
-80
PBS
Insulin
Std Sedentary
HF Sedentary
HF Exercise

Figure 6: Exercise improves blunted Akt activation in skeletal muscle of high-fat-fed mice.

Tissues were harvested following insulin stimulation at the end of the 4-week study. Following a 3 hour fast were administered a single intraperitoneal injection of sterile PBS or insulin (0.1 U/kg) 30 minutes before sacrifice. In all cases except the high-fat exercise group in the sciatic nerve, insulin-stimulation significantly increased Akt activation compared to PBS. Proteins were measured by Western blot. Representative images and quantification of group means. Insulin-stimulated Akt activation in the lumbar dorsal root ganglia (A), sciatic nerve (B), and gastrocnemius (C). Phosphorylated (ser473) Akt band intensities were normalized to total Akt.

Figure 6


A. Dorsal Root Ganglia
Std Sed
HF Sed
HF Ex
Pbs
Ins
p(ser473)Akt
total Akt
~ 60 kDa
pAkt/total Akt
B. Sciatic Nerve
p = 0.055
C. Gastrocnemius
PBS
Insulin

exercise effectively attenuated the deleterious effects of the high-fat diet on insulin signaling in muscle (Figure 6C). However, high-fat-fed exercised mice still had significantly lower Akt activation than sedentary standard mice, thus 4 weeks of exercise did not completely restore Akt activation to normal levels (Figure 6C).

5. Discussion

Here, for the first time, we report the effects of a high-fat diet and exercise on insulin signaling in neural tissues. In addition, our data indicates the potent ability of exercise to modify negative metabolic changes associated with diabetes and neural complications in a pre-diabetic model of painful diabetic neuropathy.

High-fat feeding clearly had detrimental metabolic effects in sedentary mice. Consistent with previous reports in the literature, a high-fat diet induces pre-diabetes characterized by elevated glucose, hyperinsulinemia, and systemic insulin resistance (increased HOMA-IR) in sedentary mice [23, 41]. Importantly, in the current study, exercise powerfully prevented each of these high-fat diet induced metabolic derangements.

Although insulin resistance is a key feature of type 2 diabetes and has been well described in type 2 diabetes [116, 277, 281, 282] and documented in type 1 diabetes [112, 114], there are few studies investigating insulin resistance in the peripheral nervous system. Blunted insulin signaling in the DRG from diabetic animals has recently been reported in the literature [117, 132]. Moreover, neuronal insulin resistance has recently been suggested as a potential mechanism involved in the pathophysiology of diabetic neuropathy [109]. As we have recently

discovered that a high-fat diet induces mechanical hyperalgesia in mice, we were interested in whether neuronal insulin resistance accompanied these behavioral changes.

Experiments in this study quantified Akt activation after insulin stimulation, which is a common and accepted method used to evaluate insulin signaling and insulin resistance in the peripheral nervous system and other peripheral tissues. Although the high-fat diet did not significantly impair insulin signaling in the DRG or sciatic nerve in sedentary mice, the fold change in Akt activation between PBS and insulin was lower in high-fat-fed (2.4 in DRG, 9.5 in sciatic) compared to standard diet fed sedentary mice (2.7 in DRG, 14.8 in sciatic) in both of these tissues. In the sciatic nerve, the reduction in insulin signaling is likely not significant due to the high standard error in the standard diet sedentary group. These data suggest that a high-fat diet does not impair insulin signaling in neural tissues to the extent that it does in muscle, but it appears theses tissues are headed toward of insulin resistance and there are dietary effects. If the study had been carried out for 8 weeks, decreased insulin signaling would potentially be significant in the neural tissues. Despite subtle changes in Akt activation in the peripheral nervous system, the high-fat diet and exercise had potent effects on insulin signaling in muscle.

In the gastrocnemius, impaired Akt activation was partially restored by exercise in high-fat-fed mice. The results in this study were consistent with previous reports that exercise improves high-fat diet-induced insulin resistance in muscle [149, 283, 284]. Importantly, exercise training also improves muscle insulin resistance in type 2 diabetic patients [155]. Increased glucose uptake via enhanced insulin stimulated Glut4 translocation is a key mechanism responsible for exercise-induced improvements in insulin sensitivity that has been documented in the literature [285, 286]. However, this is the first time the effects of a high-fat diet on neuronal insulin signaling in

sedentary and exercised mice have been studied, thus potential mechanisms underlying neural insulin resistance have not been elucidated in this high-fat model.

Behavioral changes in mechanical sensitivity were not yet significant after four weeks of high-fat feeding in this cohort of animals. However, the initiation of mechanical hyperalgesia in high-fat-fed sedentary mice typically occurs somewhere near the 4-week time point and is well established after 8 weeks of a high-fat diet, thus these results were not surprising. We purposely chose to examine peripheral nervous system insulin sensitivity after short-term high-fat feeding to determine if insulin resistance occurs near the onset of behavioral changes. Although there were no statistically significant changes in mechanical sensitivity, percent paw withdrawal was 65% in standard diet sedentary mice while it was 70% in high-fat-fed sedentary mice at week 4, suggesting that the HS group is headed toward mechanical hypersensitivity. In addition, insulin signaling was not signficantly impaired in the DRG or sciatic, but at the sciatic nerve appears to be headed toward insulin resistance. The lack of significant changes in insulin signaling and mechanical sensitivity coupled with the appearance that these parameters are moving in the direction of neural insulin resistance and mechanical hyperalgesia, suggest these changes may track together in high-fat-fed sedentary mice. If mechanical hyperalgesia developed in conjunction with neural insulin resistance, it is plausible to suggest that neural insulin resistance may play a role in high-fat diet induced mechanical hyperalgesia.

Previous studies in our laboratory (Groover, unpublished data) indicate exercise reverses high-fat diet induced mechanical hyperalgesia, but this does not occur until after 8-12 weeks of exercise in high-fat-fed mice. Thus, it was not surprising that exercise did not significantly impact mechanical sensitivity at these early time points in this short-term study.

In conclusion, voluntary running wheel exercise was effective at preventing detrimental changes in body weight, glucose, insulin, and insulin signaling in high-fat-fed mice. Although peripheral nervous system insulin signaling was not significantly altered by a high-fat diet or exercise, exercise attenuated insulin resistance in the muscle of high-fat-fed mice. Results from this study emphasize the importance of exercise in preventing or reversing metabolic derangements and neural complications associated with pre-diabetes.

CHAPTER 6

Conclusions

Diabetic neuropathy is the most common and debilitating complication of diabetes. Although the majority of diabetic patients will develop some form of peripheral neuropathy, 30-40% of diabetic patients do not develop neuropathic symptoms. It is unclear why some patients escape sensory complications. In addition, the reason why some patients suffer from painful symptoms while others present with painless symptoms remains elusive. There are also increasing reports of nondiabetic patients with pre-diabetes and nondiabetic individuals with normal glucose levels who develop neuropathy. It is clear that peripheral sensory neurons are damaged via several metabolic insults that are primarily due to hyperglycemia. However, the dichotomous presentation of neuropathic symptoms, the fact that not all diabetic patients develop neuropathy, and the prevalence of neuropathy among pre-diabetic patients points to additional etiological factors besides hyperglycemia. An evolving body of clinical evidence has established dyslipidemia as an independent risk factor for the development of neuropathy. Because the majority of dyslipidemia cases in adult humans can be attributed to excess energy and fat intake, diet may play an important role in the development of diabetic neuropathy.

The overall purpose of this study was to better understand the role of diet and dyslipidemia on the development and progression of diabetic neuropathy and identify potential mechanisms underlying the pathogenesis of high-fat diet induced neuropathy. Studies in this dissertation evaluated the effects of a high-fat diet on parameters used to characterize neuropathy including sensorimotor behavior, nerve conduction velocity, and epidermal innervation in nondiabetic and diabetic mice. In addition, experiments were performed to assess mitochondrial dysfunction, inflammation, and insulin sensitivity as potential pathological mechanisms responsible for the effects of high-fat feeding on sensory changes.

Chapter 2: Phenotypic Changes in -Fat Diet in Diabetic Neuropathy Induced by a High Diabetic C57Bl/6 Mice

Although clinical evidence and rodent studies suggest that dyslipidemia and/or diet plays an important role in the development and progression of diabetic neuropathy, the effects of a high-fat diet in conjunction with type 1 diabetes has not been studied. Here, for the first time, we report the effects of a high-fat diet and dyslipidemia on neuropathic symptoms in nondiabetic and STZ-induced type 1 diabetic mice.

The findings from this study were both surprising and groundbreaking for the diabetic neuropathy field. Interestingly, STZ-induced diabetic C57Bl/6 mice fed a high-fat diet developed dyslipidemia and a painful neuropathy (mechanical hyperalgesia) instead of the insensate neuropathy (mechanical insensitivity) that normally develops in this mouse strain. This finding is particularly important because it suggests that diet may modulate diabetic neuropathy phenotype. Nondiabetic mice fed the high-fat diet also developed dyslipidemia and painful neuropathy. In terms of mechanical sensitivity, the effect of the high-fat diet appears to overpower the effect of diabetes in this mouse strain. STZ-diabetic mice first develop hyperalgesia, rather than loss of sensation. Additionally, STZ-diabetic mice developed mechanical hyperalgesia with a similar time course and severity as the nondiabetic mice. These data suggest that influence of diet or dyslipidemia is potent, and perhaps over the course of several years, diet may have a profound influence on neuropathic symptoms in nondiabetic or diabetic humans. Furthermore, these results provide new insight to suggest diet may play a role in determining the neuropathy phenotype in human diabetic patients.

It is noteworthy to mention that although high-fat-fed nondiabetic and diabetic mice were both dyslipidemic, the NdHF group had elevated total cholesterol and LDL-C while the DbHF

group had elevated triglycerides. Although the majority of large clinical population studies cluster derangements in one or many lipid variables together as dyslipidemia [33, 287, 288], one mutlti-center trial specifically demonstrated that elevated triglycerides, but not elevated total cholesterol, was correlated with loss of sural myelinated nerve fiber density in diabetic patients with neuropathy [287]. Thus, there is very little evidence to base conclusions on which lipid variables are more likely to affect neuropathy development and phenotype.

Since high-fat diets induce weight gain in humans and rodents, it could be argued that increased body weight or body fat rather than lipid status is driving the effects of high-fat diet induced neuropathy in the current study. However, high-fat-fed diabetic mice weighed less than both the nondiabetic mice fed the standard diet and diabetic mice fed the standard diet, suggesting that body weight is not the driving factor behind high-fat diet induced mechanical hyperalgesia. In support of our findings, Wiggin and colleagues demonstrated elevated triglycerides, but not BMI, were correlated with detrimental changes in peripheral sensory nerve fibers [287].

In the current study, both diabetic groups exhibited thermal hypoalgesia but these sensory deficits were not exacerbated by the high-fat diet. It is not uncommon to observe opposite changes in mechanical and thermal sensory behavior in high-fat-fed nondiabetic rodents Consistent with our results, previous studies of high-fat-fed rodents report mechanical hyperalgesia and thermal hypoalgesia [5, 23, 41]. It has been proposed that the opposing mechanical and thermal behavioral changes in high-fat-fed rodents may be attributed to severe large myelinated fiber loss and morphological changes in conjunction with insignificant changes in small myelinated and unmyelinated fibers [41]. In the current study, sensory and motor nerve conduction velocity deficits were only present in diabetic mice that consumed the high-fat diet

compared to high-fat-fed nondiabetic mice. Thus, diabetes alone did not significantly impair nerve conduction velocity. These results are in contrast to some previous studies that report diabetes-induced deficits in sensory and motor nerve conduction velocities [5, 192, 289, 290]. However, there were only 3 animals in the DbStd group because several animals did develop diabetes after STZ-injection. It is also plausible to suggest that slowed sensory and motor nerve conduction velocities in the DbHF group compared to NdHF are in part due to increased sensory and motor nerve conduction velocities in nondiabetic mice fed a high-fat diet. We expect that high-fat feeding in growing nondiabetic mice may enhance myelination in large nerve fibers, and consequently result in the significant increases in sensory and motor nerve conduction velocities observed here.

Finally, nitrotyrosine, a marker of oxidative stress, was quantified in the DRG using immunohistochemistry. Surprisingly, the high-fat diet did not increase oxidative stress in high-fat-fed nondiabetic or diabetic mice. In contrast, nitrotyrosine expression was increased in medium sized neurons in diabetic mice on the standard diet. These negative results weaken the hypothesis that lipid induced neuronal injury is due to increased oxidative stress via a LOX-1 dependent mechanism.

It would be interesting to investigate the effects of lipid lowering therapies such as statins to determine if dyslipidemia mediates the high-fat diet induced effects on sensorimotor behavior. Interestingly, data from NHANES 1999-2004 indicated statin use was associated with increased incidence of peripheral neuropathy in United States adults over the age of forty [291]. An additional future direction for this work would include investigating the effectiveness of omega-3- fatty acid supplementation to prevent or reverse high-fat diet induced neuropathy. Positive outcomes from future studies could lead to dietary modifications and/or increased use of

treatments that improve dyslipidemia (i.e. omega 3 fatty acid supplementation, statins, or exercise) as therapeutic interventions for patients with painful diabetic neuropathy.

Chapter 3: Mitochondrial Function in Diabetic Neuropathy

Due to heavy reliance on mitochondrial energy production, neurons are especially sensitive to disruptions in mitochondrial dysfunction. Diabetes-induced hyperglycemia is associated with and may cause mitochondrial dysfunction in rodent models of diabetic neuropathy [63, 64, 66, 224, 292]. Consequently, mitochondrial dysfunction has been proposed as a key mechanism that contributes to neuronal dysfunction and damage in diabetic neuropathy [22, 224, 292]. In addition, previous reports suggest high-fat diet induced dyslipidemia leads to oxidative stress that can damage mitochondria [5]. We hypothesized that a high-fat diet in conjunction with STZ-induced type 1 diabetes would exacerbate mitochondrial dysfunction. In addition, we suspected neuronal mitochondrial dysfunction might be responsible for the robust behavioral phenotype associated with high-fat feeding. Here, we performed experiments to assess mitochondrial function in the lumbar DRG of nondiabetic and STZ-diabetic mice fed a standard or high-fat diet. The methodology utilized in this study was particularly novel. All previous studies that examined mitochondrial function in DRG in rodent models of diabetic neuropathy performed the mitochondrial respiration experiments using primary cultured DRG. This was the first time mitochondrial respiration has been quantified in whole, undisrupted DRG and their peripheral axonal processes. Furthermore, we used a novel high-fat/STZ-induced type 1 diabetic mouse model of diabetic neuropathy. This was the first time mitochondrial function has been assessed in this unique diabetic neuropathy model.

Results from this study indicated basal mitochondrial respiration was reduced while mitochondrial proteins Complex III subunit Core-2 and VDAC were increased in diabetic mice fed the standard diet. These results were consistent with previous studies reporting impaired mitochondrial respiration in lumber DRG from diabetic mice fed a standard diet. Despite increases in two mitochondrial proteins in the DbStd group, diabetes did not significantly alter the expression of proteins indirectly or directly involved mitochondrial function and biogenesis (UCP2, PGC-1a, Sirt1, pAmpk, pAkt, or pmTor). Surprisingly, the high-fat diet did not significantly impact mitochondrial respiration or the mitochondrial proteome in nondiabetic or diabetic mice. Because high-fat feeding was not associated with significant changes in mitochondrial respiration or the mitochondrial proteome, it is unlikely that mitochondrial dysfunction is driving the robust behavioral phenotype observed in high-fat-fed nondiabetic or diabetic mice.

Reduced basal respiration in conjunction with increases in expression of oxidative phosphorylation subunit (Complex III subunit Core-2) and VDAC could point to compensatory mitochondrial biogenesis in the DbStd group compared NdStd. However, an alternative theory could potentially explain these results. As described in Chapter 3, diabetic mice fed the standard have increased β-hydroxybutyrate levels, indicative of ketosis. In diabetes or starvation, excess Acetyl coenzyme A (CoA) is produced from fatty acid oxidation and is subsequently converted to acetoacetate and β-hydroxybutyrate in the liver. These ketones are released in the blood and transported to peripheral tissues where they can be used as alternate fuel sources rather than glucose. β-hydroxybutyrate can be converted to acetoacetate or utilized directly as an energy source. Acetoacetate is metabolized to produce two acetyl CoA that can enter the citric acid cycle.

In order to breakdown acetoacetate to acetyl CoA, succinyl CoA is required in the first step of the reaction. In the citric acid cycle, succinyl CoA is converted to succinate. Next, succinate is converted to fumarate and $FADH_2$ is produced. When an animal is in ketosis, succinyl-CoA is used to breakdown excess acetoacetate, resulting in excess succinate production. Consequently, additional succinate is available for the conversion to fumarate in the citric acid cycle and the result is increased $FADH_2$ [293]. Therefore, increased acetoacetate breakdown can result in increased $FADH_2$ production in the citric acid cycle [218].

Since Complex II of the electron transport chain relies solely on $FADH_2$ as an electron donor, increased availability of $FADH_2$ can result in increased electron flux through Complex II [218]. Consequently, the downstream components of the electron transport chain participate in a reduced proportion of the electron transport. Complex III is directly downstream of complex II thus increasing electron transport through complex II might reduce electron transport at complex III, thus stimulating an increase in Complex III subunits as observed in the diabetic mice fed the standard diet. The fact that the mice in DbHF group were also in ketosis but mitochondrial respiration and expression of mitochondrial oxidation phosphorylation proteins were not different from nondiabetic mice could weaken the "increased Complex II flux" argument. Although the reduction in basal mitochondrial respiration was not statistically significant in DbHF, it was 31% lower than NdStd while basal respiration in the DbStd group was similar and 36% lower than DbStd and this difference was statistically significant. Although statistically insignificant, a minimal reduction in mitochondrial respiration may still be physiologically relevant.

In order to confirm or refute the "increased Complex II flux" theory proposed above, (to explain increased expression of Complex III Core-2 in DbStd), mitochondrial respiration

experiments should be performed in permeabilized DRGs or isolated mitochondria in order to evaluate complex specific respiration. It would be interesting and worthwhile to perform these experiments in freshly isolated (rather than cultured) DRG to further characterize the effect of diabetes and the high-fat diet on mitochondrial function in this novel model of diabetic neuropathy.

Chapter 4: The Role of Inflammation in High-Fat Diet-Induced Hyperalgesia

Diabetes has been characterized as an inflammatory disease. Both type 1 and type 2 diabetic patients exhibit a state of chronic low-grade inflammation [87] characterized by increased pro-inflammatory mediators in the circulation [294, 295], liver [296], adipose [77], immune cells [85, 100, 297], and/or muscle [298]. Hyperlipidemia has also been shown to cause chronic inflammation and contribute to the pathogenesis underlying type 2 diabetes [299]. Inflammation has recently been proposed as a mechanism contributing to the pathogenesis of diabetic neuropathy [74, 102, 255] and has been specifically implicated in painful neuropathy [93, 256]. However, it is questionable whether inflammation is simply linked with diabetic neuropathy due to the role of inflammation in diabetes or if inflammation is a causative factor driving detrimental changes in the nervous system.

Obesity is also associated with a state of chronic low-grade inflammation [81, 82, 295, 300] and high-fat feeding in rodents increases pro-inflammatory cytokine and chemokine expression in serum and peripheral tissues [124, 190, 301]. Because the high-fat-fed animals exhibited robust mechanical hyperalgesia and have increased body weight, we expected to observe spinal and/or peripheral inflammation in these animals. Here, we hypothesized that a

high-fat diet induced central and/or peripheral inflammation in conjunction with glial activation in the spinal cord, thus resulting in chronic mechanical hyperalgesia. Experiments performed in this study assessed spinal and peripheral inflammation along with microglial activation in nondiabetic and diabetic mice fed a standard or high-fat diet. This was the first time central and peripheral nervous system inflammation has been assessed in this novel high-fat/STZ-induced type 1 diabetic model of diabetic neuropathy.

In contrast with our hypothesis, diabetes nor the high-fat diet altered cytokines or chemokines associated with inflammation in the lumbar spinal cord. In addition, microglial activation in the lumber dorsal spinal horn was not different among groups. Furthermore, TNF-α expression was not significantly increased by diabetes or high-fat feeding in the serum, sciatic nerve, gastrocnemius, or footpad. Our findings were inconsistent with one previous study that reported increased lumbar spinal TNF-α and IL1-β in STZ-induced diabetic rats [257]. However, inflammation marker levels peaked at 4 weeks post-STZ. We did not assess spinal inflammation until 8 weeks post-STZ. It is plausible that compensatory mechanisms might return inflammatory markers to normal levels after the initial response, which would make inflammation undetectable at later stages of diabetes. The effects of a high-fat diet on inflammatory mediators in high-fat-fed STZ-induced diabetic mice have never been studied before, thus we have no previous results with which to compare our findings in this specific treatment group.

Although these results suggest spinal nor peripheral inflammation is driving the development of hyperalgesia after 8 weeks of high-fat feeding in nondiabetic and diabetic mice, further investigation is needed before completely ruling out inflammation as a potential mechanism underlying high-fat diet induced neuropathy. It is important to note that we have not yet examined inflammatory mediators in the DRG or adipose. The DRG could be a key site

where inflammation might induce mechanical hyperalgesia in the hindpaws. Moreover, it would be beneficial to evaluate inflammation at other key intervals along the time course of behavioral changes. Studies designed to assess central and peripheral nervous system inflammation just prior to and at the initiation of hyperalgesia may reveal early inflammatory changes that are ameliorated by compensatory mechanisms after several weeks. Although TNF-α is the molecule with most evidence to suggest a role for inflammatory cytokines in diabetic neuropathy,

a more complete profile of inflammatory mediators should be examined in the serum and peripheral tissues. Finally, it would be interesting to determine if administration of an anti-inflammatory agent prevents or reverses high-fat diet induced hyperalgesia. Perhaps inflammatory changes in high-fat-fed mice are physiologically relevant, but minimal enough to be undetectable with the current methodology.

Chapter 5: The Effects of High-Fat Feeding and Exercise on Insulin Sensitivity

Emerging evidence indicates insulin resistance occurs in neurons as it does in insulin-dependent tissues in the setting of diabetes [117, 132]. Because insulin is a key neurotrophic factor that supports neurite outgrowth [121, 122], regeneration [123-125], and maintenance of mitochondrial function [21, 66], impaired insulin signaling due to insulin resistance could lead to neuronal damage and degeneration. Therefore, peripheral nervous system insulin resistance has recently been proposed as an etiological factor involved in the dying-back type neuronal degeneration that occurs in diabetic neuropathy [117].

In this study, experiments were designed to determine if peripheral nervous system insulin resistance accompanied mechanical hyperalgesia in high-fat-fed nondiabetic mice, a model of pre-diabetes. In addition, we tested the effects of exercise on metabolic variables and insulin resistance in high-fat-fed mice. Importantly, voluntary running wheel exercise ameliorated the detrimental effects of the high-fat diet on body weight, circulating glucose and insulin levels, and HOMA-IR. In addition, impaired Akt activation in muscle was partially rescued by exercise in mice that consumed a high-fat diet. Although there were no statistically significant changes Akt activation in neural tissues, the pattern of the fold change from PBS to insulin-stimulated Akt activation was suggestive of a trend toward blunted insulin signaling in high-fat sedentary mice compared to standard diet sedentary mice. If the study was carried out a few weeks longer, perhaps significant peripheral nervous system insulin resistance would be present.

Previous reports from our laboratory and others indicate that nondiabetic rodents fed a high-fat diet develop mechanical hyperalgesia [23, 30, 41]. Studies in our laboratory have consistently indicated that mechanical hyperalgesia first begins to appear in C57Bl/6 mice after 4 weeks of a high-fat diet ([30] and unpublished data). Thus, we specifically chose the 4-week time point in order to assess insulin sensitivity at the onset of behavioral changes. In the current study, high-fat-fed sedentary mice did not display statistically significant mechanical hyperalgesia after 4 weeks of high-fat feeding, but percent paw withdrawal threshold was 71% which was 13% higher than baseline. Similar to Akt activation, despite the lack of statistical significance, the behavior appears to be headed toward mechanical hyperalgesia in the high-fat sedentary group. Plus, we have consistently observed significant mechanical hyperalgesia after 4 weeks of high-fat feeding in multiple cohorts of mice.

Considering the lack of statistically significant changes in insulin signaling and mechanical behavior, it would be interesting to assess these variables after 5, 6, 7, and 8 weeks of high-fat feeding to determine if behavioral changes occur near the same time as neural insulin resistance. Interestingly, insulin-stimulated Akt activation and neurite outgrowth is significantly decreased in DRG cultures from diabetic *ob/ob* mice (type 2 diabetes model) that exhibit mechanical hyperalgesia [132]. Although it has been purported that insulin resistance occurs in type 1 diabetes [302], to our knowledge, no one has examined insulin signaling in the peripheral nervous system in type 1 diabetic rodents. Moreover, to elucidate whether neural insulin resistance is more closely associated with a painful or painless phenotype of diabetic neuropathy, it would be beneficial to characterize insulin signaling in STZ-induced rodent model of insensate diabetic neuropathy.

Impact on the Field

Altogether, this body of work was transformational for the field of diabetic neuropathy. For the first time, we demonstrated that a high-fat diet can potently modify neural symptoms by switching neuropathy phenotype from insensate to painful. These results shed light on why some diabetic patients experience painless symptoms while others present with painful symptoms. The knowledge gained from these findings provides a springboard for clinical investigations examining the relationship between diet composition and neuropathy development and phenotype. Furthermore, these studies suggest dietary intervention may be a useful therapeutic approach for preventing or reversing symptoms of diabetic neuropathy.

Additionally, we report findings from three studies in which the novel high-fat/STZ-induced type 1 diabetic model of diabetic neuropathy was used for the first time to investigate potential mechanisms (including oxidative stress, mitochondrial function, and neuronal insulin resistance) that may contribute to diet-induced changes in neural symptoms. Although many of the results were negative, these studies provide valuable guidance for future investigations to focus when searching for mechanisms underlying high-fat diet induced neuropathy and the relationship between dyslipidemia and neuropathy risk in diabetic patients.

Finally, we were the first to test the effects of exercise on neuronal insulin signaling in high-fat-fed mice. Exercise prevented deleterious effects on circulating glucose and insulin levels and insulin resistance in high-fat-fed mice. Results from this study reinforce the importance of exercise in preventing or reversing metabolic derangements and neural complications associated with diabetes. These data provide strong support to suggest clinicians should incorporate exercise intervention as a novel therapeutic to prevent and treat diabetic neuropathy.

Importantly, some key achievements from the work summarized in this dissertation advanced the diabetic neuropathy field. First, new animal models were developed to better parallel the human condition. These animal models included STZ-induced type 1 diabetes with dyslipidemia, pre-diabetes, and pre-diabetes with exercise intervention. In addition, novel approaches were utilized to explore mitochondrial dysfunction in diabetes. Specifically, mitochondrial respiration was measured in freshly isolated and undisrupted DRG and peripheral axons. Finally, experiments tested novel mechanisms associated with high-dietary fat that may underlie hyperalgesia, including inflammation and insulin resistance. In conclusion, this dissertation provides strong evidence for the influence of lifestyle factors including diet and physical activity on metabolic defects and neural complications associated with diabetes.

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CHAPTER 7

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